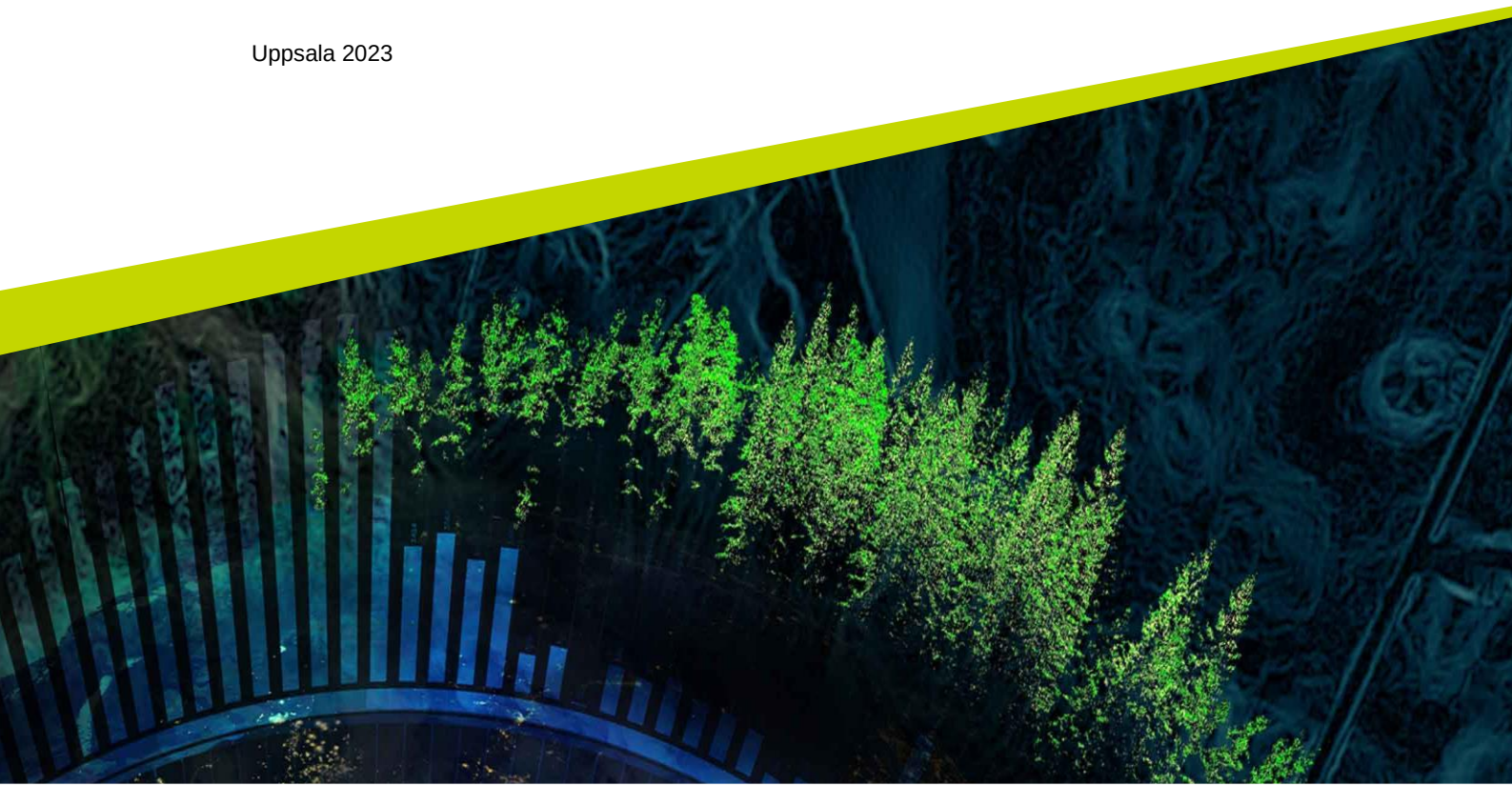
Predicting Gastrointestinal Adverse Events in Dogs Treated with Chemotherapeutic Medication

Emmie Andersdotter

Independent Project • 30 credits
Swedish University of Agricultural Sciences, SLU
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Uppsala 2023



Predicting Gastrointestinal Adverse Events in Dogs Treated with Chemotherapeutic Medication

Emmie Andersdotter

Supervisor:	Henrik Rönnberg, Swedish University of Agricultural Sciences, Department of Clinical Sciences
Assistant supervisor:	Sara Saellström, Swedish University of Agricultural Sciences, Department of Clinical Sciences
Examiner:	Ingrid Ljungvall, Swedish University of Agricultural Sciences, Department of Clinical Sciences

Credits:	30 credits
Level:	Second cycle, A2E
Course title:	Independent Project in Veterinary Medicine
Course code:	EX1003
Programme/education:	Veterinary Medicine Programme
Course coordinating dept:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2023

Keywords:	Calprotectin, cancer, canine, chemotherapy, cytostatic medication, dog, gastrointestinal toxicity, gastrointestinal adverse events, gut microbiota, influential factors, questionnaire

Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Veterinary Medicine Programme

Abstract

Despite significant advances in the treatment of cancer in canine patients, gastrointestinal toxicity still remains a relatively common finding after chemotherapeutic treatment. These adverse events may cause a fundamental reduction in the patient's quality of life. To improve the well-being of the patients, and to minimize the risk of adverse events following chemotherapy, the mechanisms and reasons behind the development of adverse events have to be understood. There are several possible factors that might affect the risk of developing chemotherapy-induced gastrointestinal toxicity, however, there are currently no standardized methods for reviewing, mapping or measuring these factors within the field of study. The use of questionnaires, in combination with non-invasive biomarkers, could therefore potentially be a stress-free and favourable way of investigating the correlations between chemotherapy and gastrointestinal adverse events as well as a way of predicting which animals that are at risk for gastrointestinal toxicity.

A prospective study at University Animal Hospital (UDS) in Uppsala, Sweden, was performed with the ambition of investigating the connection between chemotherapeutic treatment and the development of gastrointestinal toxicity. The main aim of this study was to find possible influential factors leading to the development of gastrointestinal adverse events after chemotherapeutic treatment. The study was divided into two questionnaire-based parts with questions directed to owners of dogs with a cancer diagnosis. The first questionnaire reviewed potential influential factors in the everyday life and diet of the dog which could be related to the development of chemotherapy-induced adverse events. This questionnaire also examined the frequency of gastrointestinal events as well as concurrent illnesses and treatments. The second questionnaire focused on the occurrence and assessment of gastrointestinal toxicity in dogs treated with chemotherapeutic medication based on VCOG-CTCAE (version 2).

A total of eight dogs with cancer of different ages, sexes, and breeds were included in the study. According to the owners, 87% (n=7) of the dogs had experienced some form of mild gastrointestinal disturbance without the need for supportive therapies during the last year. Three of the eight canine patients continued with chemotherapeutic treatment and could be assessed through the second questionnaire. In total, 67% (n=2) dogs experienced different grades of gastrointestinal adverse events (loss of appetite, diarrhoea) within three to five weeks after their first chemotherapeutic treatment.

The questionnaires show promise to be used in studies with similar aims, possibly in combination with the analysis of biomarkers. However, due to the small study population, the results from this study may not be representative of a larger population. It is not possible to determine whether the gastrointestinal events that were reported in this study were caused by the cytostatic agents or if they had another aetiology. Therefore, further studies must be performed regarding potential influential factors as well as to investigate the actual clinical utility of the questionnaires. Further studies regarding the use of non-invasive biomarkers such as calprotectin and/or gut microbiota may also be of importance to examine the association with the development of gastrointestinal adverse events.

Keywords: Calprotectin, cancer, canine, chemotherapy, cytostatic medication, dog, gastrointestinal toxicity, gastrointestinal adverse events, gut microbiota, influential factors, questionnaire

Table of contents

1. Introduction

Neoplastic diseases are important in dogs, and cancer is one of the leading causes of death in canine patients (Adams *et al.*, 2010; Bonnett *et al.*, 2005; Hoffman *et al.*, 2018; Veterinary Cancer Society, 2022). Treatment of cancer in our companion animals has greatly improved over the last few decades and several different therapeutic options are now available.

Some neoplastic diseases can be treated with chemotherapy. However, this form of treatment may be associated with unwanted side effects (i.e., adverse events) from several organ systems (MacDonald, 2009; Tomiyasu *et al.*, 2010; Vail, 2009). One of the most common adverse events is gastrointestinal toxicity, with clinical signs such as vomiting, nausea, and diarrhoea (Vail, 2009). The prophylactic use of medication may reduce the risk of unwanted side effects following treatment, and this preventative treatment is in many instances sufficient for maintaining a good quality of life (Pang & Argyle, 2016). In some patients, however, cancer treatment may lead to the development of adverse events which have the ability to severely impair their quality of life (Chavalle *et al.*, 2022). On some occasions, the toxicity can become so severe that the disadvantages outweigh the advantages, making the continuation of treatment indefensible without intervention.

To improve animal welfare, and to reduce the risk of gastrointestinal adverse events during chemotherapeutic treatment, there is a need for a wider understanding of the correlation between cytostatic agents and gastrointestinal toxicity and the mechanisms behind it. The possibility to predict which animals may be at risk for developing gastrointestinal adverse events may be of great value in order to customise treatment protocol and optimise the use of prophylactic medication. Furthermore, it allows the clinician to focus intensive pre-treatment on certain predisposed animals. This is of great importance since reducing the risk of these adverse events can be vital and life-changing for canine cancer patients.

In addition to being of great importance for canine patients, the potential use of questionnaires or biomarkers, such as calprotectin or the gut microbiota, may also be useful in human medicine to evaluate the risk for adverse events after cancer treatment (Hoffman *et al.*, 2010; Thamm, 2019). Dogs have been suggested as an

ideal model for understanding human cancer in translational medicine due to their physiological similarity to humans, their similar clinical presentation of cancer, and their reactions to its treatments (Hoffman *et al.*, 2010). Progress in veterinary oncology may therefore not only be of importance for animal cancer patients, but also for potential advances in human oncology

The main aim of this study are the piloting of two questionnaires for predicting and assessing gastrointestinal adverse events. The first questionnaire reviews possible influential factors in the everyday life of the canine patient that might relate to the development of gastrointestinal adverse events after chemotherapeutic treatment. The second questionnaire is focused on gastrointestinal toxicity in canine patients after chemotherapy, as well as their well-being and quality of life after treatment. Secondary aims are to provide a literature overview regarding the connection between cytostatic medication and the development of gastrointestinal toxicity. Additionally, a literature overview regarding possible biomarkers in faeces will be performed in order to assess their potential use in combination with the questionnaires to facilitate the identification and assessment of canine patients at risk for developing gastrointestinal adverse events after chemotherapeutic treatment.

2. Literature review

2.1 Cancer in canine patients

2.1.1 Cancer-related mortality in dogs

The risk of death following cancer in dogs is relatively high**.** It is estimated that neoplastic diseases are the cause of death in one of four dogs**,** although the absolute mortality is not fully known due to difficulties in obtaining reliable and consequent data (Reif, 2007).

A study of mortality in dogs in the United Kingdom reported cancer as one of the most commonly reported causes of death, with proportional mortality of 27% in the purebred dogs included in the study (Adams *et al.,* 2010). Earlier studies have also found a relatively high proportional rate of cancer-related deaths, ranging from 14.5% in a Danish study by Proschowsky *et al.* (2003) to 23% in a necropsy study by Bronson (1982). Bonnett *et al.* (2005) investigated the mortality in 350,000 Swedish insured dogs and found that 18% of death cases in canine patients under 10 years of age were tumour related. It is also well recognized that there are differences between breeds and their risk for developing cancer due to their genetic composition or size (Bronson, 1982; Dobson, 2013; Proschowsky *et al.*, 2003). The incidence rate and mortality of cancer also increase with age and are affected by sex (Adams *et al.*, 2010; Bonnett *et al.*, 2005; Bronson, 1982; Dobson, 2013; Proschowsky *et al.*, 2003).

2.2 Cancer treatment in canine patients

Cancer treatment in dogs has increased significantly during the last 20 years (Pang & Argyle, 2016). The main therapeutic options for canine neoplasia include surgery, chemotherapy, and radiation therapy, used alone or in combination with other treatments (Gustafson *et al.*, 2013; Wolfesberger *et al.*, 2012). During the last decades, advances in cancer immunotherapy treatment have also been made,

making immunotherapeutic treatment a possible option for managing or treating cancer in both human and veterinary patients (Morrison, 2010; Regan *et al.*, 2016).

2.2.1 The choice of cancer therapy

Therapy choice is made based on multiple aspects about the patient and their type of cancer. Some factors to take into consideration are tumour type, tumour cell sensitivity, histologic grade, stage of the disease, patient's age, concurrent morbidities, and tolerance to side effects as well as the decision of the owner (Gustafson *et al.*, 2013). Many things may influence the owner's decision on treatment. Some owners are concerned with the potential negative impact on the patient's quality of life due to the possible development of severe treatment-related side effects. This concern often arises due to knowledge of chemotherapeutic treatment in human medicine, which more often is associated with severe side effects (Elting *et al.*, 2003). Further reasons for a specific choice of therapy, or for excluding a therapy, can be the owner's economic abilities. Some treatment options can be expensive, and the possibility of prolonged or supplementary treatment as well as the risk or need for hospitalisation following treatment could be a risk some owners aren't willing to take. Furthermore, client enthusiasm also plays a substantial role in the choice of initiating and continuing cancer therapy (Vail, 2009).

The main goal of cancer treatment is complete remission**,** i.e., to eradicate and cure cancer permanently. However, this is often not entirely possible. The main focus of the treatment given to canine patients is often rather to stop further cancer development and spread of malignant neoplastic cells, and to increase the duration and quality of their lives (Gustafson *et al.*, 2013).

2.2.2 Chemotherapy

Surgical options for removing and limiting the spread of neoplasia are most common and effective for local cancer types, early-stage disease, and tumours with low metastatic potential (Farese *et al.*, 2013). However, chemotherapeutic medication may be an effective and suitable option for cancer therapy depending on the therapeutic intent and goal. Cytostatic therapy may be used as a primary treatment or as adjuvant therapy following surgical or radiational treatment of a tumour. It may also be used prior to other treatments in hope of reducing tumour size to facilitate surgical removal (Gustafson *et al.*, 2013). Effective chemotherapeutic therapy in animals with sensitive tumours may result in extended long-term survival and a greater quality of life (Gustafson *et al.*, 2013; Vail, 2009).

Most cytostatic agents target rapidly dividing cells by interfering with the cell cycle, with the aim of shrinking, destroying or stopping the growth of cancer cells. This is performed by affecting DNA replication and synthesis and in extension the cellular division (Gustafson *et al.*, 2013; MacDonald, 2009). However, the chemotherapeutic medication cannot differentiate between healthy and normal, highly proliferative cells and neoplastic cells (Gustafson *et al.*, 2013; MacDonald, 2009; Vail, 2009), which increases the risk of possible adverse events.

2.2.3 Adverse events after chemotherapeutic treatment

Definition & mortality

The Veterinary Cooperative Oncology Group (VCOG, 2004; 2016) has published a consensus document of common terminology criteria for adverse events following chemotherapy in canine and feline patients, recently reviewed and updated by LeBlanc *et al.* (2021). This consensus document provides a way to standardise the classification and grading of adverse events. VCOG (Leblanc *et al.*, 2021) defines an adverse event (AE) as "*any unfavorable and unintended sign, clinical sign, or disease temporally associated with the use of medical treatment that may or may not be considered related to the medical treatment".* The adverse events can be graded on a scale of 1 through 5 based on the severity of the event, with 1 being mild symptoms and 4 being life-threatening adverse events that require urgent interventions. A grade 5 adverse event is often defined as death and is therefore seldom used for grading treatment-related side effects. Although there are standardised ways to score the severity of adverse events in both human and veterinary medicine, the method for evaluating these events is not uniform or standardised (Giuffrida *et al.*, 2017). The use of a methodical and systematic way to identify and report these adverse events is imperative to accurately assess the frequency of toxicity in treatments and studies and to facilitate the comparison between different studies.

Most patients tolerate chemotherapeutic treatment without any, or minimal, adverse events (MacDonald, 2009). The majority of patients enjoy a good quality of life during treatment and the owner's opinion following this therapy is often positive (Cunha *et al.*, 2016). It is generally described that 1 in 4 patients treated with chemotherapeutic medication will experience adverse events and less than 5% have a serious event that requires hospitalisation (Bowles, 2010; Cunha *et al.*, 2017). Some research has been carried out on the incidence of adverse events in canine patients treated with certain protocols of chemotherapeutic agents. Cunha *et al.* (2017) performed a retrospective study reviewing adverse events in 292 canine chemotherapy patients and found that 20-25% of these patients developed adverse events. The majority (83%) of these adverse events were considered mild (grade 1)

toxicities. In another retrospective study on 155 dogs treated with chemotherapy, adverse events were reported at least once in 80% of patients and severe adverse events were observed in 32% of patients (Chavalle *et al.*, 2002). A possible reason for the large difference in incidence may be that the use of prophylactic medication wasn't standardised in their studies. Whilst these studies give an insight into the incidence of adverse events in dogs, the general frequency of mild and severe adverse events is, to this author's knowledge, rarely described.

Common adverse events following chemotherapy

Adverse events following chemotherapy are one of the major challenges in cancer treatment and can be seen in many different organ systems due to the cytostatic agents' affinity for highly proliferative cells (Gustafson *et al.*, 2013; MacDonald, 2009; Vail, 2009). Different chemotherapeutic agents can affect organ systems differently, although the most commonly seen adverse effects are bone marrow toxicity and gastrointestinal toxicity.

Bone marrow toxicity following chemotherapy

Bone marrow toxicity is the most common side effect of treatment with cytostatic agents (Gustafson *et al.*, 2013; MacDonald, 2009; Vail, 2009, Tomiyasu *et al*., 2010). The chemotherapeutic agents often cause myelosuppression resulting in peripheral blood cytopenias. Therefore, thrombocytopenia, anaemia, and different types of granulocytopenias are all possible chemotherapeutic consequences, but neutropenia is the absolute most common finding (MacDonald, 2009; Tomiyasu *et al.*, 2010). Lower neutrophil concentration in the body may allow enteric bacteria to enter the bloodstream, with fever, septicaemia, and life-threatening sepsis as a possible result (MacDonald, 2009).

The suppression nadir, i.e., where the blood cell count is at its lowest following chemotherapeutic drug administration, varies with individual drugs. However, in dogs and cats, the suppression nadir is often seen 5-10 days after treatment (MacDonald, 2009). When using combination protocols containing different types of cytostatic agents, it is of great importance to ensure that the nadirs do not coincide to avoid additive effects on bone marrow suppression. Patients who prior to treatment suffer from myelosuppression in any bloodline are generally at greater risk of a more extensive suppression following chemotherapy. To ensure a reduced risk of serious cytotoxic effects on the bone marrow following treatment, it is standard practice to perform hematologic and chemistry blood work prior to treatment (Backlund, 2021).

Although suppressive effects of bone marrow toxicity can have negative effects on patient well-being, the suppression has at the same time been found to have positive

effects on survival time after treatment. Wang *et al.* (2015) found that the patients experiencing the most prominent bone marrow suppression had longer remission and survival times than patients that did not experience bone marrow toxicity. Therefore, they also suggested chemotherapy-induced neutropenia as a useful marker for the prediction of treatment response.

Gastrointestinal toxicity following chemotherapy

The gastrointestinal tract is often highly affected by chemotherapeutic agents due to the high mitotic rate of crypt cells (Vail, 2009; Wang *et al.,* 2015). Therefore, is gastrointestinal toxicity one of the most frequent and important adverse events following chemotherapy in both human and canine patients (Chavalle *et al.*, 2022; Gustafson, 2013; Cunha *et al.*, 2017; Elting *et al.*, 2003**;** Vail, 2009; Tomiyasu, 2010).

Common adverse events due to gastrointestinal toxicity are vomiting, nausea, diarrhoea, inappetence, and anorexia. There are many other potential side effects of chemotherapy affecting the gastrointestinal tract, ranging from an increased amount of flatulence or intestine inflammation to ileus and megaoesophagus. A complete list of possible gastrointestinal adverse events after chemotherapy in canine patients can be found in VCOG-CTCAE (version 2, Leblanc *et al.,* 2021). The adverse events can also be of different severity, extending from asymptomatic or mild to severe and life-threatening.

A gastrointestinal adverse event caused by cytostatic treatment is chemotherapy-induced nausea and vomiting, abbreviated CINV (Hesketh, 2008; Vail, 2009). Generally, CINV can be acute or delayed. The acute form of CINV is defined as nausea or vomiting within the first 24 hours after chemotherapy. Acute CINV may develop due to specific therapeutic agents or arise if an infusion is performed too rapidly (Gustafson, 2013; Vail, 2009). Delayed CINV occurs 2-5 days after chemotherapy treatment. The delayed form of CINV is most frequently seen, often presenting as inappetence, nausea, and vomiting (Vail, 2009). The pathological mechanism of CINV is not fully clarified, although it is suggested that the acute form of CINV can be caused by damage to the intestinal epithelial cells (Logan *et al.*, 2008) and/or efferent stimulation of the chemoreceptor trigger zone (Hesketh, 2008; Vail, 2009). Currently, antiemetic medication is often included in the chemotherapy treatment protocols since prophylactic treatment of CINV is far superior to the treatment after symptoms have occurred (MacDonald, 2009; Vail, 2009).

Diarrhoea after cytostatic treatment is also a fairly common adverse effect, affecting up to 60% of human chemotherapy patients (Stein *et al.,* 2010). The same number

in canine patients is not completely known. CID may cause dehydration, malnutrition, and hospitalisation; however, the severity and frequency of the events depend on the type of drug and schedule for administration (Stein *et al.*, 2010). The pathophysiological reason for the development of CID is not completely understood, but several reasons are suggested. Through research on animal models it has been found that a possible cause of CID is cytotoxic effects on intestine crypt cells and gastrointestinal lining (Gibson *et al.*, 2006; Richardson & Dobish, 2007). Following crypt cell and mucosal damage, the absorptive surface area and other intestinal functions may be altered, resulting in an inability to absorb water from the intestines, resulting in diarrhoea (Gibson *et al.*, 2006). Secondly, the normal composition of the intestinal microflora may be altered due to cytostatic treatment. The damage to the crypt cells may facilitate opportunistic bacterial adherence to damaged tissue, which may cause dysbiosis and/or overgrowth of opportunistic bacteria (Gibson *et al.*, 2006). Richardson and Dobish (2007) also suggest changes in the enzyme balance as a possible cause for the development of CID. Damage to the bowel mucosa may also result in a higher risk of sepsis since this secondarily enables bacterial translocation over the intestinal wall. This may arise secondary to mucosal damage and concurrent neutropenia facilitates bacterial translocation (Vail, 2009). Preventing and managing CID is therefore essential for maintaining an acceptable quality of life during cancer treatment.

Incidence of gastrointestinal adverse events

The incidence of gastrointestinal adverse events varies with the use of different chemotherapeutic agents. Tomiyasu *et al.* (2010) evaluated adverse events in 40 dogs with different treatment protocols using VCOG-CTCAE (version 1.0) and found an incidence of gastrointestinal adverse events (grade 2 or higher) in 8.1%, 17.0%, and 50% for treatment with doxorubicin, cyclophosphamide, and vincristine respectively. The severity of the symptoms also varied with the treatment protocol. Tomiyasu *et al.* (2010) also found that patients with a higher stage of disease encountered a higher risk of suffering from adverse events following treatment. In another study by Chavalle *et al.* (2021) reviewing the medical records of 155 dogs receiving chemotherapy with different treatment protocols, a total of 14.8% suffered from gastrointestinal events. Cunha *et al.* (2017) studied adverse events in 292 dogs and instead found vomiting in 21% of patients, diarrhoea in 20% of patients, and inappetence in 20% of patients. These results suggest a fairly high risk of developing gastrointestinal toxicity following chemotherapeutic treatment.

2.3 The gut microbiota in dogs

A factor suggested for the development of gastrointestinal disturbances following chemotherapy is alterations in the composition of the normal gut microbiota, i.e., dysbiosis (Gibson *et al.,* 2006; Zitvogel *et al.,* 2017).

2.3.1 The normal gut microbiota

The microbiota is commonly defined as the spectrum of living microorganisms currently present within a defined environment (Marchesi *et al.,* 2015), including bacteria, archaea, protists, protozoa, fungi, and algae (Berg *et al.,* 2020). Non-living microorganisms (e.g.viruses, plasmids, phages) are typically not included in this definition (Dupré *et al.,* 2009), but can be included in other definitions (Suchodolski, 2011). The word microbiota is often mistaken for the word microbiome, despite being different terms. The microbiome is instead defined as a "*characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties"* as written by Whipps *et al.* in 1988. This definition has since been further endorsed in a commentary article from a workshop of leading international experts in the microbiome field, written by Berg *et al.* (2020). This article suggests the definition of the microbiome as the microbiota along with their "theatre of activity", referring to their microbial structures and metabolites, their genome, and the surrounding environmental conditions (Berg *et al.,* 2020; Marchesi *et al.,* 2015).

Composition of the normal gut microbiota

The different parts of the gastrointestinal tract hold different kinds of micro-organisms due to anatomical and physiological reasons (Suchodolski, 2005). Because of this, various types, species, and strains of microorganisms have certain, specialised functions in the gastrointestinal tract and can utilise host nutrients while providing metabolites available for host uptake (Suchodolski, 2011). The micro-organisms composing the microbiota can interact with the host in different manners; they can be symbiotic (i.e. benefitting from each other by being mutualistic), commensals (i.e. benefitting from each other while not disturbing one another), pathogens (i.e. capable of causing disease), or parasitic (i.e. using the host to survive without giving anything in return) (Desselberger *et al.,* 2018).

The knowledge of the gut microbiome in canine patients is much less complete than in humans. In the last few decades, however, the development and use of metagenomic sequencing methods have allowed progress to be made in the field of mapping the canine gut microbiota (Gavazza *et al,* 2017; Simpson *et al*., 2022; Suchodolski *et al.,* 2008a, Suchodolski *et al.,* 2008b, Suchodolski *et al.,* 2011; Swanson *et al.,* 2011).

Bacteria in the normal canine gastrointestinal tract

Overall, the healthy gut microbiota in all mammals is predominantly constituted of bacteria (Swanson *et al.*, 2011). It is estimated that the gastrointestinal tract in dogs contains 10^{12}-10^{14} microbial cells (Suchodolski, 2016). The total bacteria count and diversity in the gut microbiota in canine patients varies along the gastrointestinal tract, with increased bacterial numbers aborally along the digestive tract (Suchodolski, 2011). The concentration of bacteria also varies between the gut mucosa and gut lumen (Mentula *et al.*, 2005). It is approximated that the stomach microbiota in dogs contains **10^1-10^6** colony-forming units (CFU) of bacteria per gram or millilitre of content (Johnston, 1999). The canine duodenum and jejunum generally accommodate a relatively small number of bacteria, typically 10^5 cfu/g, but ranging up to 10^9 cfu/g (Johnston, 1999; German *et al.*, 2003), while the ileum contains greater bacterial numbers, approximated to 10^7 cfu/g (Suchodolski, 2011). The ileum also contains a more heterogeneous microbiota. The colon of the dog holds the highest bacterial count, ranging from 10^9 to 10^{11} cfu/g of faeces (Mentula *et al.*, 2005).

The normal canine intestinal tract is home to several hundreds of bacterial phylotypes (Suchodolski *et al.*, 2009; Swanson *et al.*, 2011). According to Suchodolski (2011), the most common bacterial types in dogs include *"Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria".* Together, these bacterial groups constitute over 99% of all gut microbiota. The same results have been found in a metagenomic study conducted by Swanson *et al.* in 2011. The general distribution of the population of bacteria along the canine intestine has also been investigated in several studies by Suchodolski (2009; 2011). In the small intestine, aerobic or facultative aerobic bacteria predominated**;** the duodenum and jejunum held bacteria such as Clostridia, Proteobacteria, and Lactobacillales whereas anaerobic bacteria could be found in greater abundance in the ileum and colon, predominated by Bacteroidales (Firmicutes, Bacteroides, and Fusobacteria).

Other microorganisms in the canine gastrointestinal tract

Fungi, archaea, protozoa, and viruses also reside in the gastrointestinal tract of most mammals**,** although their role, influence, and interaction with their host remain uncertain (Suchodolski, 2011). It is also unclear what effects some of these microorganisms have on the development and continuation of gastrointestinal diseases (Suchodolski, 2011).

Yeast and moulds have been found in the faeces of healthy Beagle dogs (Mentula *et al.*, 2005) and a study by Suchodolski *et al.* (2008b) showed that several types of fungi can be found in the intestine in up to 76% of dogs. Suchodolski *et al.* (2008b)

also suggested that opportunistic fungi with pathogenic abilities may be found in the canine gastrointestinal tract.

Archaea, a type of bacterial-looking organism without a cell nucleus, can also be found along the gastrointestinal tract of dogs where they represent approximately 1% of the total microbiota (Swanson *et al.*, 2011). These commensals are usually found in the intestine of ruminants but can also be found in the intestine of other mammals (Swanson *et al.*, 2011).

A diverse community of viruses can also be found in the canine intestine. Recent metagenomic studies found that viral sequences answer to less than 0.4% of the canine microbiota, with over 99% being associated with bacteriophages (Swanson *et al.*, 2011). Kempf *et al.* (2010) reported that the most common viral findings in the canine gastrointestinal tract are rotavirus, coronavirus, and parvovirus.

2.3.2 The normal function of the gut microbiota

The gut microbiome plays a crucial role in many bodily functions and overall host health of all mammals (Suchodolski *et al.*, 2012; Suchodolski, 2016). In all mammals, the gut commensals primarily aid in digestion and nutrient metabolism. Some members of the microbiota have the ability to convert and metabolise dietary products through different processes such as fermentation and hydrolysis to facilitate nutrient uptake of the host (Young, 2017). The ability to ferment dietary substrates into short-chain fatty acids (SCFA) (acetate, propionate, and butyrate) is, for example, significant for energy production and is of importance for gastro-intestinal health and the gut immune system (Jandhyala *et al.*, 2015; Suchodolski, 2011). Some microbes also have the spectrum of activity to synthesise important cofactors or vitamins and may also be able to facilitate the metabolism of drugs and toxins (Young, 2017). In addition to this, non-pathogenic bacteria also contribute to the integrity of the intestinal barrier and structure of the gastrointestinal tract by acting as a barrier against pathogenic microorganisms. They also affect intestinal permeability and can help prevent the colonisation of pathogenic microorganisms (Jandhyala *et al.*, 2015; Suchodolski, 2011).

The intestinal microbiome also has an essential role in the development of the innate and adaptive immune system in both humans and animals (Suchodolski, 2011; Jandhyala *et al*, 2015). It has an immunomodulatory effect on the body by working together with the innate and adaptive immune system both systematically and locally, e.g. by contributing to the development and shaping of the normal lymphoid tissue in the digestive tract (Jandhyala *et al.*, 2015). Host microbiota and immune system work together to maintain a balanced inflammatory response to develop tolerance towards the non-pathogenic bacteria and host tissue in the gastrointestinal

tract while enacting an inflammatory response in the presence of pathogenic bacteria required for eradication (Abt *et al.*, 2012; Petersen & Round, 2014). If this balance isn't upheld, there is a risk of unwanted immune responses.

2.3.3 Dysbiosis

As previously mentioned, the normal gut microbiota and host cooperate to maintain a homeostatic, well-balanced immune response to keep the host healthy. If the microbial composition somehow is altered, there is a risk for negative effects, both on the diversity of microbes and the immune system function (Petersen & Round, 2014). This event of microbial imbalance is referred to as *dysbiosis.*

Petersen & Round (2014) suggest three categories of dysbiosis: (1) Loss of beneficial microorganisms, (2) Reduced diversity, and (3) Pathobiont expansion. Disruptions to the normal composition may cause a diversity loss, mainly affecting the favourable commensals, which allows potentially harmful bacteria (i.e. pathobionts) to grow and divide excessively (Petersen & Round, 2014). For example, an increased abundance of certain microbes, such as members of the family *Enterobacteriaceae,* has been found to be a marker of dysbiosis in both human and dog patients (Rivera-Chavez *et al.,* 2017; Vazquez-Baeza *et al.,* 2016). Pathobionts are usually not pathogenic in immune-competent hosts since the beneficial bacteria keep them at a low level. However, the domination in the gut of these pathobionts may cause inflammation and pathology (Petersen & Round, 2014).

Broadly defined, dysbiosis refers to any alteration in the composition and/or concentration of the microbiota in relation to the composition found in a healthy individual (Petersen & Round, 2014; Suchodolski, 2016). Studies in human patients have implied individual and interindividual variations in normal microbial composition in the gastrointestinal tract due to genetics, age, diet, host environment, the use of antibiotics, as well as the use of pro- and prebiotics (Turnbaugh *et al.*, 2007; Schwabe & Jobin, 2013**;** Petersen & Round, 2014). This has also been studied in animal patients. Different types of dietary habits have in studies been found to impact the abundance of certain types of bacterial groups, primarily affected by the overall macronutrient composition (recently reviewed by Pilla & Suchodolski, 2020). This can be further endorsed by Simpson *et al.* (2002) and Middlebos *et al.* (2010) who suggested that exogenous factors, such as diet and fibre content, to some extent can influence microbiota in canine patients. In contrast, AlShawaqfeh *et al.* (2017) found no significant impact of dog food containing different amounts of fibre, fat, or protein content on the gut microbiota in dogs with inflammatory enteropathy compared to healthy dogs. The lack of correlation between protein

content and a higher dysbiotic index has also been found in previous studies (Minamoto *et al.,* 2015; Vazquez-Baeza *et al*, 2016).

2.3.4 The gut microbiota and cancer

Exogenous factors, as listed above, may cause alterations of the microbial composition in the gut, causing dysbiosis which can lead to inflammatory states and diseases. Studies in human and veterinary patients have shown associations between intestinal dysbiosis and gastrointestinal diseases or disorders, such as irritable bowel disease (IBD), irritable bowel syndrome (IBS), granulomatous colitis (Suchodolski *et al.,* 2012; Honneffer *et al.*, 2014, Vich Vila *et al*., 2018) as well as cancer (Gavazza *et al.,* 2017; Herstad *et al.*, 2018; Omori *et al.,* 2017*).*

In recent years, evidence suggests that the gut microbiota plays an important role in the development and pathophysiology of cancer, however, the actual causality between cancer and dysbiosis still remains unclear. Specific pathogens have been found as a possible cause of the development of some cancer types due to the acute inflammatory response that follows an infection (Rosadi *et al.,* 2016; Peek & Blaser, 2002). Overall disruptions and imbalances in the gut microbiota ecosystem and homeostasis may also play an essential role in the development of cancer, both in human and animal patients (Zitvogel *et al*., 2017). Many pathogens, primarily viruses, can promote cancer through various genetic and immunomodulatory mechanisms. In some cases, viruses may perform genomic integration to integrate viral genome into host DNA, which has been found in both human and canine cancer types (Epiphanio & Santos, 2021; Pagano *et al.*, 2004; Thaiwong *et al.*, 2018). Another route for carcinogenesis is genotoxicity, where certain pathogen strains may produce toxins by promoting genome instability, affecting host DNA integrity, and/or affecting tumour suppressor genes (Pagano *et al.*, 2004; Rosadi *et al.*, 2016). Microorganisms may also, in addition to acute inflammation, trigger chronic inflammation, which directly and indirectly can promote cancer development (Coussens & Werb, 2022; Fukata & Abreu, 2008)**.**

It is well known that the development of cancer involves many alterations in bodily functions, structures, and environments, which also affect the normal microbiota and microbiome. Therefore, evidence suggests that some diseases may not solely be attributable to single pathogens, but also to ecosystemic changes in the gut microbiota (Khan *et al.,* 2013). The normal gut microbiota is crucially involved in the normal development and function of the adaptive and innate immune systems, which are essential for the balance between inflammatory activation and tolerance (Abt *et al.,* 2012; Petersen & Round, 2014). The less stable microbial community found during dysbiosis can therefore promote overactive or impaired local,

regional, and systemic immune responses, resulting in inflammatory states which may be pro-neoplastic (Schwabe & Jobin., 2013; Khan *et al.*, 2012).

Intestinal dysbiosis in canine cancer patients has to some extent been investigated, particularly in patients with gastrointestinal tumours. When investigating changes in gut microbiota in dogs with multicentric lymphoma and comparing them to healthy dogs, Gavazza *et al.* (2017) found significant changes in microbial composition as well as a higher dysbiosis index. This suggests a possible association between systemic neoplastic disorder and effects on the intestinal microbiota. Similar findings have been made by Omori *et al.* (2017) where dogs with intestinal lymphoma and IBD were shown to have significant changes in faecal microbiota compared to healthy dogs. In addition, microbial changes with a reduction of health-promoting bacteria and an increase in potentially pathogenic bacteria have also been found in dogs with colorectal cancers (Herstad *et al.*, 2018). The effects of microbial dysbiosis on non-gastrointestinal cancers are however rarely studied (Viaud *et al.*, 2013). These abovementioned studies have described changes in the intestinal microbial composition but have not been able to distinguish whether changes in the microbiota in cancer patients are a consequence of the neoplastic changes or if they are causing them.

2.3.5 The gut microbiota and chemotherapy

Systemic chemotherapy is one of the most important and essential parts for treating some types of neoplasia in both humans and dogs. However, systemic cancer therapies can affect the gut microbiota, which in turn could influence the chemotherapeutic treatment response (Iida *et al.*, 2013). Chemotherapy may induce alteration in the normal gut microbiota composition and its diversity (Zitvogel *et al.*, 2017). In addition, dysbiosis and an overgrowth of potentially pathogenic bacteria that might follow may negatively impact the treatment response (Aarnoutse, 2019).

The importance of microbial drug metabolism has been well-known since the 1960s (Scheline, 1968), and the importance of the microbiota-driven modulation of chemotherapeutic agents is increasingly recognized (Jia *et al.*, 2008). The gut microbiota may influence the efficacy, toxicity, and effect of most classes of cytostatic agents by activating or inactivating the medication or by modifying the immune responses (Alexander *et al.*, 2017, Polk *et al.*, 2010). These modulatory effects of the gut microbiota may be direct; using deamination, demethylation, or reduction to change the chemotherapeutic agent, or indirect by competing for host enzymes or affecting the enterohepatic recycling (Wilson & Nicholson., 2017). In a study conducted by Lehouritis *et al.* (2015), findings suggest that bacterial biotransformation of different anti-cancer drugs could change the efficacy of their

anti-tumour potential both positively or negatively, increasing or decreasing the therapeutic effects. The gut microbiota may therefore, to some extent, explain differences in treatment responses between individual patients, and their toxicity profiles.

Several studies have investigated the effects of chemotherapy on the intestinal microbial composition in human patients, recently reviewed by Aarnoutse *et al.* (2019). The results from studies have all indicated that chemotherapy induces drastic changes in the microbial composition in the gut, which might negatively affect the efficacy of the treatment. Viaud *et al.* (2013) used mouse models to investigate the role of intestinal gut microbiota in chemotherapeutic treatment. They found that cyclophosphamide, a clinically important cancer drug, could alter the gut microbiota sustainably. Significant reductions of commensal bacteria (e.g. *Lactobacillus johnsonii* and *Enterococcus hirae*) could be found after 7 days of treatment with cyclophosphamide, causing substantial alterations in the intestinal microbiota. Furthermore, long-term antibiotic use caused translocation of gram-positive bacterial species and affected some adaptive T-helper-driven cell responses needed to lower the tumour burden in these mice. Similarly, Daillére *et al.* (2016) demonstrated that cyclophosphamide modified the composition of the gut microbiota and found that certain microorganisms present in the intestine were important for the anti-cancer effects of the chemotherapeutic agent. These studies indicate that a functional microbial ecosystem is important for both patient response and some immunomodulatory effects. Understanding how the gut microbiota influences and modulates chemotherapeutic treatments therefore of importance for optimisation for patients undergoing cancer treatment and their quality of life.

2.3.6 Characterisation of the gut microbiota

Cultivation has for a long time been a technique commonly used for the characterization of the microbial composition in the gastrointestinal tract (Mentula *et al.*, 2005, Simpson *et al.*, 2002). This approach is specifically useful for the detection of specific pathogens and for testing the microbial sensitivity of different antibiotics. The use of the cultivation method is limited since it is highly biased toward a few phylogenetic groups, primarily bacteria and fungi (Overmann *et al.*, 2017). It is also recognised that most intestinal bacteria cannot be cultivated using classical cultivation methods (Fraher *et al.*, 2012; Suchodolski, 2016). The majority of the microbial species in a sample can therefore not be properly identified, and many previous studies using culture-dependent approaches have therefore very likely underestimated the diversity in the intestine, in both animal and human samples (Suau *et al.*, 1999; Suchodolski *et al.*, 2004; Suchodolski, 2016). Cultivation is therefore not suited for assessing more complex environments such as the gut microbiota. During the last few decades, the use of molecular techniques

for characterising more diverse microbial environments has increased dramatically. This technique is based on the identification of 16S ribosomal RNA found in most bacteria and has greatly improved the speed and efficiency of microbial profiling in comparison to its predecessor (Suau *et al.,* 1999; Suchodolski *et al,* 2004)**.**

2.4 Faecal calprotectin

Calprotectin is a calcium- and zinc-binding protein complex consisting of two proteins from the S100-family. The protein was first described by Fagerhol *et al.* in 1980 and has since then been studied frequently**.** With current techniques, the protein can now be measured in several biological fluids and tissues, e.g. including serum and faeces (Pathirana *et al.,* 2018; Enderle *et al.,* 2022). Calprotectin concentration in faeces seems to be more specific for gastrointestinal diseases (Pathirana *et al.,* 2018; Enderle *et al.,* 2022), possibly due to neutrophil migration in inflamed gastrointestinal tissue (Pathirana *et al.,* 2018).

Calprotectin is primarily expressed in neutrophil granulocytes, but it is also, although to a lesser extent, found in macrophages and monocytes (Bjarnason, 2017; Fagerhol *et al.,* 1980; Pathirana *et al.,* 2018). In neutrophils, the protein accounts for approximately 60% of the cytosolic protein fraction, and higher concentrations of the protein can therefore be found in the presence of neutrophils (Bjarnason, 2017; Pathirana *et al.,* 2018). Neutrophil granulocytes are common effector cells in the early phase of the inflammation cascade and will release cytosolic granules, including calprotectin, on the site of chemoattraction (Bjarnason, 2017; Boussac *et al.,* 2000; Stríz *et al.,* 2004). Therefore, the amount of calprotectin released in the inflamed area can be perceived as a reflection of the number of participating neutrophils in the inflammatory event (Bjarnason, 2017; Yui *et al.,* 2003). Furthermore, calprotectin has an additional effect on the innate immune response since it acts as a mediator in inflammation by acting as a damage-associated molecular pattern protein, also known as alarmins (Fengming *et al.,* 2014; Heilmann, 2017). These mediators may contribute to the recruitment of other inflammatory cells, such as monocytes, to the inflammatory site (Catalán *et al.,* 2011). The protein also has effects on the neutrophils themselves by facilitating adhesion and phagocytosis of neutrophils (Fengming *et al.,* 2014; Heilmann, 2017). Several independent studies have shown that calprotectin is associated with both acute and chronic inflammation (Bjarnason, 2017; Enderle *et al.,* 2022, Heilmann & Allenspach., 2017; Pathirana *et al.,* 2017). Thereby, this concludes that calprotectin can play a regulatory role in the inflammatory process and that calprotectin has the potential to be used as an inflammatory biomarker.

Faecal calprotectin is routinely used in human medicine as a non-invasive sensitive biomarker for inflammation in the gastrointestinal tract (Pathirana *et al.*, 2017; Bjarnason, 2017). In human patients, the protein is most commonly used to identify IBD and other intestinal diseases, as well as monitor disease activity and response to treatment. It may also be helpful for the prediction of disease relapse (Bjarnason, 2017; Smith & Gaya, 2012; Pathirana *et al.*, 2017).

In veterinary medicine, calprotectin concentration is mostly studied in patients with inflammatory gastrointestinal diseases, such as IBD or chronic inflammatory enteropathy (Grellet *et al.*, 2012; Heilmann, 2015; Otoni *et al.*, 2018). Studies have shown higher faecal calprotectin concentrations in dogs suffering from chronic diarrhoea than in healthy control dogs and they have also found a significant correlation between histological lesions in the gastrointestinal tract and higher faecal calprotectin levels. Furthermore, Otoni *et al.* (2018) also suggested correlations between the severity of disease and higher levels of inflammatory biomarkers in faeces. In conclusion, these findings indicate that higher levels of calprotectin may correlate to the severity of gastrointestinal disease and thereby inflammation, which supports the use of faecal calprotectin as a non-invasive method for the evaluation of gastrointestinal inflammation. Further studies are however required to evaluate the clinical utility of faecal calprotectin in different inflammatory states in veterinary medicine.

To this author's knowledge, there are no studies reviewing changes in the concentration of faecal calprotectin prior to and after chemotherapeutic treatment in canine patients.

2.4.1 The analysis of calprotectin

As stated above, calprotectin has the potential to be used as a non-invasive biomarker for diagnosis and severity in canine patients suffering from enteropathies. Although calprotectin can be found in both animal and human patients, the use of human immunoassays for calprotectin analysis in animal patients has failed, which makes the method for analysis of the protein in animals less available. Enderle *et al.* (2022) suggest that this may be caused by the human assay using monoclonal antibodies against human calprotectin. Therefore, the use of species-specific antibodies is needed for analysis. Enderle *et al.* (2022) and Heilmann *et al.* (2008) have both evaluated methods for the analysis of canine calprotectin, both in faeces and serum. Heilmann *et al.* (2008) validated a radioimmunoassay as a sensitive and accurate approach for the quantification of calprotectin while Enderle *et al.* (2022) instead verified the turbo immunoturbidimetric assay as a precise and reproducible assay for analysis of faecal calprotectin in cats and dogs. Recently, a new sandwich

enzyme-linked immunosorbent assay technology was available for the analysis of calprotectin in serum, plasma, and other biological fluids (Abbexa, 2022)

Faecal calprotectin, and calprotectin found in other biological fluids, is highly resistant to most types of degradation, including degradation from intestinal and pancreatic enzymes and bacteria (Bjarnason, 2018). The protein is also stable, withstanding over 7 days at room temperature (Bjarnason, 2018). The stability of the protein and the possibility of non-invasive sample collection could potentially make faecal calprotectin a good biomarker for the evaluation of inflammatory states in the gastrointestinal tract.

3. Material and Methods

3.1 Literature review

The relevant data for the literature review was obtained by searches in the databases "Web of Science", "PubMed", "Science Direct", "Google scholar", and "Primo". Search words were used in various compositions. The most frequently used words were: "Canine", "Dog", "Chemotherapy", "Chemotherapeutic", "Cytostatic", "Adverse Event(s)", "Side effects", "Gastrointestinal", "Gut microbiota", "Intestinal microbiota", "Microbial composition", "16S rRNA" and "Calprotectin". Additional search words were used less frequently and will therefore not be mentioned. Articles from both human and veterinary medicine were used. A few review articles were used due to the lack of relevant articles on the topic.

3.2 Study design

A prospective study was performed at University Animal Hospital (UDS) in Uppsala, Sweden, with the main aim of finding potential influential factors in the lives of canine patients resulting in the development of gastrointestinal toxicity after chemotherapeutic treatment. In the prolongation of this study, the gut microbiota and faecal calprotectin will be analysed prior to and after chemotherapeutic treatment in order to investigate their clinical utility as biomarkers for gastro-intestinal toxicity. The results from the analysis of the biomarkers will then be interpreted along with the answers from questionnaires.

All medical records of dogs with visits to the oncology clinic at the University Animal Hospital (UDS) between September 2022 and November 2022 were reviewed. Patients that were possible candidates for chemotherapeutic treatment were contacted. As a standard procedure, the owners of these dogs were contacted and were sent information about the study through email. If the contact information regarding email did not exist, owners were contacted through UDS's normal channels (i.e. through text message). Informed consent was obtained from all owners before enrolment in the study.

3.2.1 Study population

The inclusion criteria for participation in the study were to visit the oncology clinic at UDS and to have a cancer diagnosis without any previous treatment with chemotherapeutic medication. Dogs receiving their first chemotherapeutic treatment had the possibility of additional participation in the study at their return visit. For further participation, normal routine bloodwork and return visits after three to five weeks were required. Patients not fulfilling these criteria were excluded. Dogs were also excluded from the second part of the study if for some reason their treatment was ended before their first return visit, if the collection of faeces needed for the study was unsuccessful or if they chose to end their participation in the study.

3.2.2 Study data & clinical variables

In the first questionnaire, the clinical variables collected through medical records and questions answered by the owner were breed, sex, age, weight, and feeding habits. Further questions were asked concerning gastrointestinal events during the last year (mainly focusing on frequency and episodes of vomiting and diarrhoea), current comorbidities and/or illnesses as well as any concurrent treatments and supportive therapies. This questionnaire contained both open-ended and “yes” or “no” answers. The full questionnaire can be found in Appendix 1.

For the patients undergoing cytostatic treatment, medical records were reviewed between the first and second visit to identify any changes in treatment protocol, contact with the veterinary clinic and/or the possible need for hospitalisation as well as the reason for this.

The patients receiving chemotherapeutic treatment were asked to answer a second questionnaire, presented at their first return visit. This survey focused on gastro-intestinal events following chemotherapeutic treatment. Any changes in weight since the last visit were noted. This questionnaire collected information about the dogs' well-being and activity level after cytostatic treatment. The activity levels were graded from 1 to 4 as follows: 1, normal activity; 2, mild fatigue; 3, moderate fatigue; 4, severe fatigue. The quality of life was graded from 1 to 3 where 1 is normal, 2 is mildly decreased and 3 is severely decreased quality of life. The questionnaire also collected information about any new or terminated medication or supportive treatments, the need for hospitalisation or visits to veterinarians during treatment, and changes in feeding habits. The main focus of the second survey was signs of gastrointestinal toxicity, graded based on VCOG-CTCAE (version 2, 2021). The full questionnaire can be found in Appendix 2.

3.2.3 Evaluation of gastrointestinal adverse events

The presence of gastrointestinal adverse events following chemotherapy was evaluated based on their divergence from normal dogs. The signs of adverse events were identified, and the severity was graded based on the criteria for adverse events from the VCOG-CTCAE guidelines, version 2 (LeBlanc *et al.*, 2021). The gastrointestinal adverse events were graded from none (0) to severe (4). Since grade 5 gastrointestinal events implied death, this grade was not assessed in this study. This second questionnaire contained both open-ended and "yes" or "no" questions as well as systematic questions with pre-defined answers derived from the VCOG-CTACE grading scheme (version 2, LeBlanc *et al.*, 2021). The prevalence, frequency, and severity of adverse events were evaluated using a questionnaire (see Appendix 2). In addition, the patient's well-being, current treatment, and contact with the hospital or need for hospitalisation were monitored through their medical records.

3.2.4 Sample collection & processing

Faecal samples were collected from all canine patients enrolled in the study on the day of their visit to the oncologic clinic. Faecal samples from dogs proceeding with chemotherapeutic treatment were collected on their first return visit to the clinic after their first chemotherapy session. The time for return visits varied between 3 to 5 weeks based on the treatment protocol and owner compliance.

Instructions for collecting and storing the sample were specified in the information sheet sent to the owners before their clinic visit. The owners were instructed to collect the faecal samples on the evening prior to, or on the day of, their visit. The collection was instructed to be performed in a way that reduced the risk of bacterial and/or environmental contamination. The faeces should then be stored at 4°C until their visit, no more than 24 hours after collection. At the clinic, the sample was retrieved, and a pea-sized portion of the faeces (objective measurement) was transferred into a container for analysis of the microbiota. The remaining faeces was resealed into a clear plastic bag for analysis of calprotectin. The respective containers were labelled with the date of collection and the patient identification. The faecal samples were then stored frozen at −20°C until analysis or further processing.

3.2.5 Sample analysis

Faecal samples were collected and prepared as described above. Neither calprotectin nor microbiota analyses could be performed within the time frame for this study but will be analysed in a larger-scaled forthcoming study. Therefore, the

following paragraphs will provide an overview of methods that will be used in future studies reviewing the same subject.

Analysis of the gut microbiota

The gut microbiota in dogs with cancer will be assessed through 16S rRNA gene amplicon sequencing. Analysation will be performed to assess types and semi-qualitative proportions of bacteria in the samples, both prior to and after treatment with cytostatic medication.

DNA is isolated from 0.2 grams of faeces using the QIAamp DNA Mini Kit (QIAGEN, GmbH, Hilden, Germany). The procedure follows the manufacturer's instructions, but an additional mechanical lysis step is added to improve the lysis of bacterial cell walls. This step is performed using 0,1 mm zirconium/silica beads (Biospec Products INC, Bartlesville, USA). Isolated DNA will then be stored at -20°C until further processing and analysis. 16s rRNA gene amplicons are generated using the primers and sequenced by Illumina sequencing. Polymerase chain reaction amplicons (PCR amplicons) (PCR; using Phusion® High-Fidelity PCR chemistry (New England Biolabs, Ipswich, MA, USA)) are then generated using universal primers (806R and 515F, amplifying parts of the 16S gene) and are assigned sample-specific barcodes. Thereafter, PCR reactions are performed after which the positive and confirmed PCR product samples are purified with Qiagen Gel extraction kit. Purified samples are then quantified and pooled into equal amounts.

Processing and sequencing of the amplicons are performed on an Illumina HiSeq platform 2500 at Novogene (Bejing, China). The pair-end sequence reads are merged with FLASH (version 1.2.7, http:// ccb.jhu.edu/softw are/FLASH/) and are assigned to different samples using the sample-specific barcodes. The sequence data is quality filtered using WIME (V 1.7.0) and any chimaera sequences are detected and removed using the UCHIME algorithm (Version 7.0.1001). The UPARSE software (version 7.0.1001) will cluster the remaining sequences to generate OTUs (Operational Taxonomic Units), classified using 97% sequence homology.

Analysis of faecal calprotectin

Evaluation of the calprotectin levels in the faecal samples will be performed by using the Abbexa Dog Calprotectin ELISA Kit (Abbexa, 2022). The kit uses a sandwich enzyme-linked immunosorbent assay technology for in vitro quantitative measurements of the canine calprotectin concentrations in serum, plasma, and other "biological fluids". In this case, faeces will be analysed. The antibodies used are

polyclonal and dog specific and developed to recognize the target at the Glu25-Glu136 amino acid sequence on calprotectin.

After storage at -20°C, the faecal samples are thawed and homogenised. The standard solution is prepared and used for serial dilutions, and the wash buffer is diluted with distilled water. Thereafter, detection reagents A and B are prepared. The reagents must be used within a maximum of 15 minutes after their preparation. Extraction and analyses are thereafter performed according to manufacturer instructions, following the standard methods for ELISA. In summary: standards, test samples, and reagents are added to the wells on the pre-coated antibody 96-well plate. The plate is covered and incubated at 37°C for 1 hour. Unbound conjugate is removed using a wash buffer. Detection Reagent A-solution is then added to each well and the plate is once again sealed and incubated for 1 hour at 37°C. The solution is then discarded, and the plates are washed three times with wash buffer. Detection Reagent B is then added to each well, and the plate is covered and incubated for 30 minutes at 37°C. After this, the liquid is discarded, and the wash process is repeated 5 times. TMB substrate is then added to each well and is mixed using gentle taps. The plate is then incubated for 10-20 minutes at 37°C. After this, a stop solution is added to each well. The intensity of the colour in the plate is noted to determine the amount of calprotectin bound on the plate. The optical density is then measured using spectrophotometric techniques at 45 nm in a microplate reader and the concentration of calprotectin is then calculated. For complete and detailed instructions, see the manufacturer manual.

4. Results

A total of 29 dogs fit the inclusion criteria and were possible candidates for enrolment in the study between September and November 2022. Of these 29 owners, 22 owners were contacted by email and 7 owners via text message through UDS channels. Of the owners contacted through the veterinary hospital (text message), four of seven owners (57.1%) enlisted in the study, while only 4 of 22 (18.1%) from the population contacted through email participated in the study. In total, 26.6% of the contacted owners chose to participate in the study. Owners participating in the first part of the study provided faecal samples from their dogs and answered the first questionnaire.

4.1 Study population

A total of eight dogs with cancer were enrolled in the study by answering the first questionnaire and providing faecal samples. Breeds included were Alaskan Malamute (n=1), Chihuahua (n=1), Ceskoslovenský Vlciak, (n=1), Siberian Husky (n=1), French Bulldog (n=1), English Springer Spaniel (n=1), Jack Russel Terrier (n=1) and mixed breed (n=1). The median age was 12.3 years (range 6.9 to 16.1 years) and sex distribution was 2 castrated male dogs, 4 intact male dogs, 1 spayed female dog, and 1 intact female dog. Of these seven dogs, three dogs met the inclusion criteria for further participation in the study reviewing gastrointestinal adverse events following chemotherapeutic treatments.

4.2 Questionnaire 1 – Canine patients with cancer

4.2.1 Dietary habits

The dietary habits of each dog were evaluated (See Figure 1). Five out of eight dogs (62.5%) were fed both commercially made dry, pelleted dog and wet dog food as their standard diet. The brands used varied. Of these five dogs, two patients (40%) also received raw or cooked meat. Two dogs (25%) were only given dry, pelleted food as their main diet. The last dog (12.5%) was only fed commercially made, wet dog food. As treats, all patients received either commercially made dog treats or

chewing bones (See Figure 2). Two of eight (25%) patients were occasionally also given leftover food meant for human consumption as a treat. Dietary supplements were given to one patient (12.5%) on a regular basis.

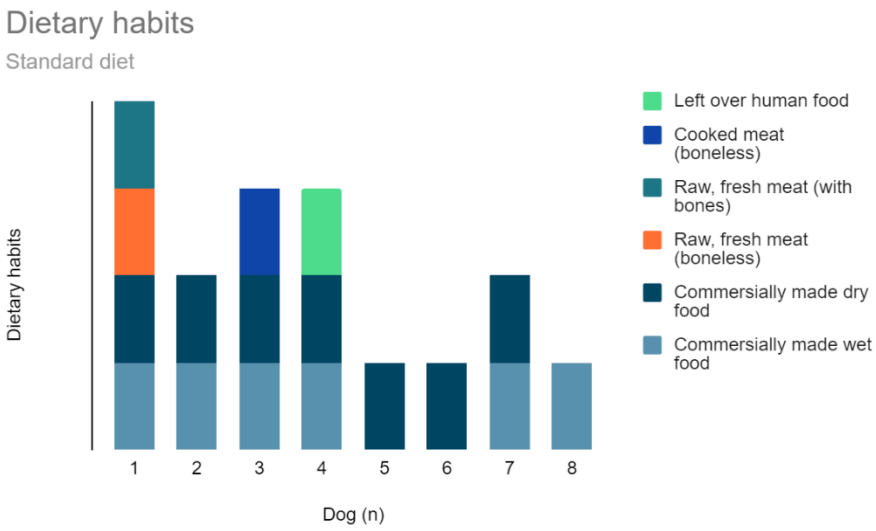


Figure 1: The different types of food included in the standard diets of eight different dogs with cancer.

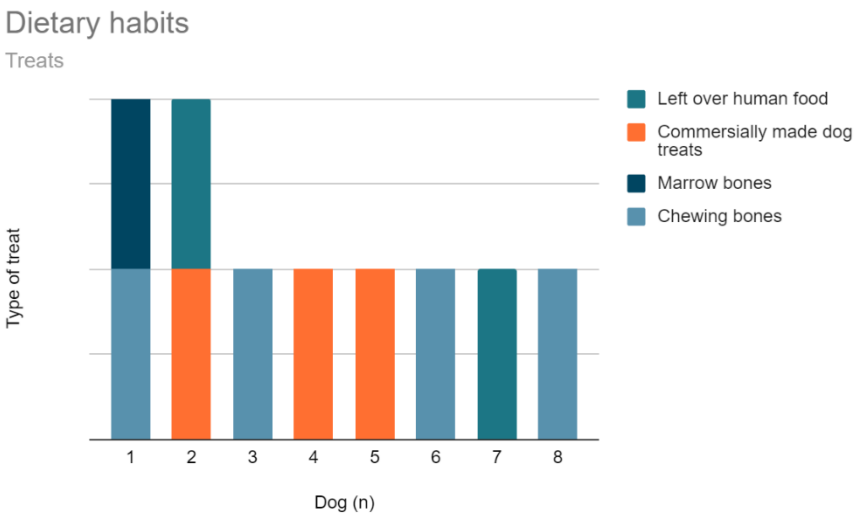


Figure 2: The different types of treats regularly given to eight different dogs with cancer.

4.2.2 Gastrointestinal events

Seven out of the eight dogs (87.5%) included in this study reported at least one episode of either vomiting or diarrhoea during the last year. One patient (12.5%) had not experienced any gastrointestinal events in the form of vomiting or diarrhoea

within the last year. Three of eight dogs (37.5%) were reported having experienced both vomiting and diarrhoea within the last year (See Figure 3). Five of the eight dogs (62.5%) reported a single episode of vomiting lasting one day and one patient (12.5%) had had several episodes of vomiting lasting one day within the last year. No patients had suffered from vomiting for more than one day for each episode. Four patients (50%) experienced diarrhoea, of which two patients only had one episode lasting one day during the last year, while two patients had several episodes of diarrhoea with a duration of two days. No reports of diarrhoetic episodes lasting over three days were reported. None of the dogs received any form of treatment (i.e rest, change of dietary habits, medication, hospitalisation, operation) due to vomiting, however, one patient received pro- and prebiotics in close proximity to diarrhoetic episodes in order to ease recovery.

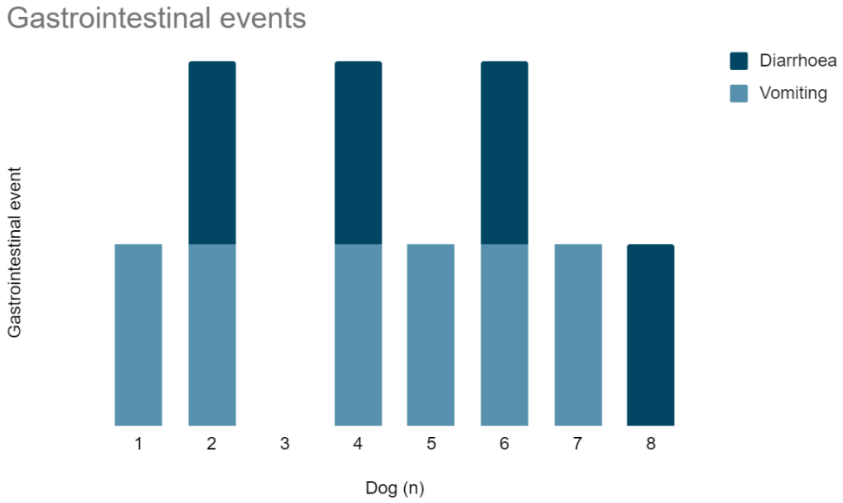


Figure 3: Incidence and type of gastrointestinal events during the last year in eight different dogs with cancer.

4.2.3 Diseases & medication

Two of the eight individuals (25%) included in this study reported comorbidities that had lasted more than one year, however, only one of these patients was treated with medication for this disease. Two of eight dogs (25%) had been treated with antibiotic medication during the last six months, one of which also had been treated with NSAID and glucocorticoids for a few weeks since the cancer was discovered.

4.2.4 Activity level & quality of life

The activity level and quality of life were evaluated by the owner based on the subjective image of their dog's well-being. Five of eight dogs (62.5%) were reported having a normal activity level (grade 1) while the owners of three of the eight dogs (37.5%) perceived the activity level of their dog as mildly decreased

(grade 2) (See Figure 4). Six of eight owners (75%) perceived their dog's quality of life as normal (grade 1) while two of eight owners (25%) reported a mildly decreased quality of life (grade 2) for their dog based on their dog's current life situation and well-being (See Figure 5).

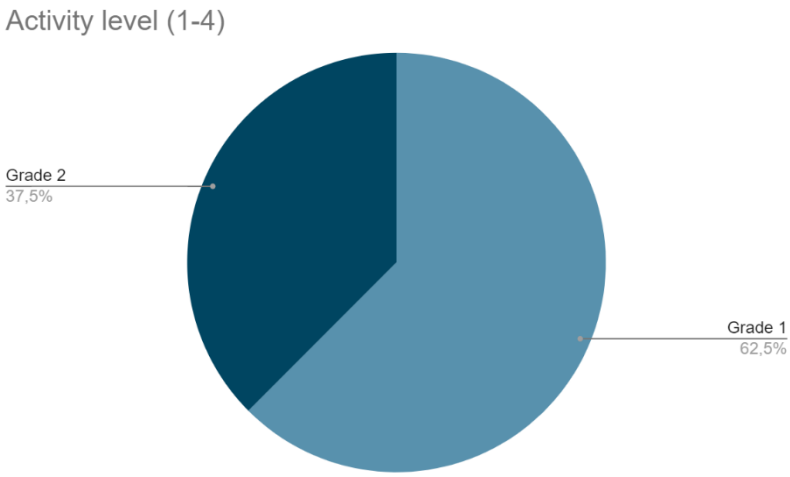


Figure 4: Current activity levels of eight different dogs with cancer graded 1-4, subjectively evaluated by the owners.

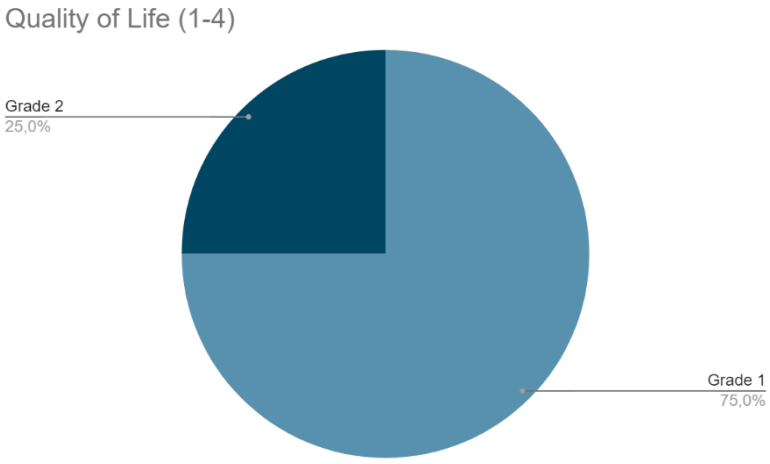


Figure 5: Current quality of life in eight different dogs with cancer graded 1-4, subjectively evaluated by the owners.

4.3 Questionnaire 2 – Canine patients treated with chemotherapy

4.3.1 Changes & adverse events

Of the eight dogs participating in the first part of the study, four dogs continued treatment with chemotherapeutic treatment. Three of those four dogs continued their participation in this study. One of the three dogs (33%) had a slight weight loss (1.9 kg, 5.7% of total body weight) leading up to the return visit three weeks later. This is reported as a grade 1 adverse event. The other patients were stable in weight during this period of time. None of the patients altered their planned medication in any way, no dietary changes were performed, and no dog was given dietary supplements.

All patients included in the second part of the study had reported at least one previous gastrointestinal disturbance in the form of vomiting and/or diarrhoea during the last year. In total, two of three (67%) dogs participating in this part of this study experienced one or more gastrointestinal adverse events within three to five weeks after their first chemotherapeutic visit. A loss of appetite was the most common adverse event seen in this study, seen in two patients (100% of patients experiencing adverse events). In the weeks leading up to their return visit, one patient (33% of the total study population) experienced a loss of appetite during $\leq$1 week following chemotherapy (grade 1 adverse event) while the second patient (33% of the total study population) experienced a loss of appetite for a duration of 1-2 weeks after chemotherapy as well as diarrhoea lasting for more than 24 hours (grade 2 adverse event). The third patient (33% of the total study population) did not experience any documented gastrointestinal adverse events but did, as mentioned above, experience a slight weight loss (grade 1 adverse event). See Figure 6 for an overview of the reported incidence of gastrointestinal events and weight loss in the three chemotherapy-treated patients. No patients with grade 3 toxicity or higher were found and none of the adverse events required medical intervention or hospitalisation. Further, no dogs needed any unplanned dose reductions of their current cytostatic drug or treatment protocol.

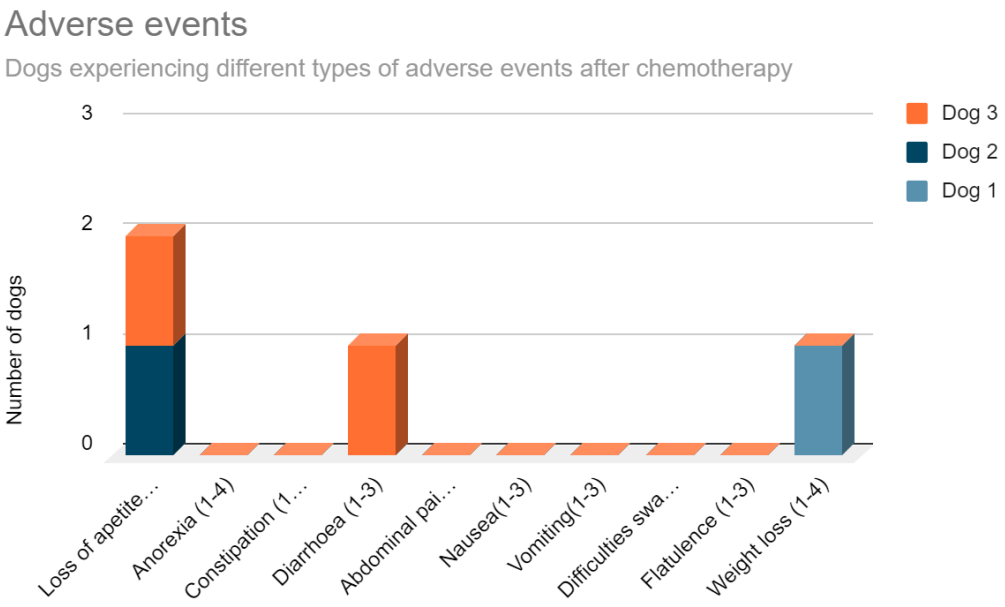


Figure 6: Incidence of gastrointestinal adverse events and weight loss in three dogs within 5 weeks after their first chemotherapy treatment.

4.3.2 Activity level & quality of life

Following chemotherapy, all patients (100%) were reported to have a normal quality of life (grade 1) based on their current life situation and well-being. Two of the three owners (67%) reported that their dogs' quality of life and activity level had improved with the treatment.

5. Discussion

The objective of this study was to find influential factors in the everyday life of canine patients that might affect the risk of developing gastrointestinal toxicity after chemotherapeutic treatment. This was performed using questionnaires. Another ambition of this study was to investigate the potential use of faecal calprotectin and the gut microbiota through a literature overview in order to anticipate the risk of developing gastrointestinal adverse events following chemotherapeutic treatment. Due to practical constraints concerning the analyses of microbial composition and calprotectin, these analyses could not be performed within the time frame for this thesis. Therefore, this study will not provide a comprehensive review of the correlations between the biomarkers and chemotherapy-induced gastrointestinal adverse events. For the same reasons, the usability of the questionnaires and their potential use in combination with the biomarkers cannot be fully examined. Analyses of the abovementioned biomarkers and a more conclusive review of their usability and their correlation with the questions asked in the questionnaires will be performed in a forthcoming larger study on this topic.

5.1 The study design

This study was conducted using two questionnaires which were answered by the owners of dogs with cancer during their visit to the veterinary clinic.

Only 26.6% of the contacted owners of dogs with cancer chose to participate in the study. A few potential explanations for this relatively low percentage of responses might be due to low interest in the study, perceived inconvenience, or confusion about the purpose of the study. An interesting result in the search for the study population was the effectiveness of different contact strategies. The majority of owners (55%) contacted through the veterinary hospital (i.e. through text message) chose to enrol in the study, while less than 20% of the owners contacted through e-mail chose the same. This suggests that a text message-based way of contact sent through official channels could be most effective to find a suitable, and hopefully larger, study population. However, owner compliance and the size of the study population are also factors to consider when interpreting these results.

5.2 The questionnaires

The questionnaires for anticipating and predicting chemotherapeutic adverse events are currently not standardised within the field of study. A standardised, objective questionnaire based on risk factors for disease and the VCOG-CTCAE document would therefore give clinicians and researchers a better opportunity for a consistent approach when collecting, comparing, and evaluating data between studies. The questionnaires that were created for this study were based on several possible influential factors found in the literature review, as well as on the common criteria for adverse events (VCOG-CTCAE, version 2, LeBlanc *et al.,* 2021) in the effort of standardisation. Although the study population was small, the owners participating in this study answered the questions as intended, which suggests that the questions were well-written and easy to understand. Although some additional questions and clarifications could be added to these questionnaires, it could be possible to use these questionnaires in future further studies with similar aims. However, to fully determine their usability, further evaluation must be performed, and a larger study population has to be used. In addition, in the prolongation of this study, the questions also have to be evaluated, and potentially be revised, in relations to the use of biomarkers or other relevant factors.

The ambition of the first questionnaire was to find influential factors in the diet or day-to-day life of the dog which might be related to an increased or decreased risk of development of adverse events after chemotherapeutic treatment. In summary, the most obvious finding to emerge from the first questionnaire was that a majority of the dogs in the study (87.5%) had experienced gastrointestinal disturbances in the form of either vomiting or diarrhoea during the last year. Three of the eight dogs (37.5%) had been afflicted with episodes of both vomiting and diarrhoea. None of the patients required intervention of any kind. These results imply that most dogs experience some form of mild gastrointestinal disturbance without need for supportive therapies. This is be supported by the available literature. It is commonly known that mild acute self-limiting diarrhoea and/or vomiting are common in the dog population, affecting approximately 15-20% of all dogs within a two-week period (Hubbard *et al.*, 2007; Candellone *et al.*, 2020). In a study by Edwards *et al.* (2004), however, the same numbers for the same clinical signs and the same amount of time varied between 1.8% and 2.2%. Despite the large difference in prevalence between the studies, it can still be concluded that it is common with a mild form of gastroenteritis in the general canine population. These studies investigated the incidence of gastrointestinal disturbances during a two-week period. The timed reviewed for gastrointestinal disturbances in this study was however a year. A higher percentage of cases is therefore expected when examining the same clinical signs during a larger amount of time, as performed in this study. Hubbard *et al.* (2007) also concluded that diarrhoea and vomiting generally have a short duration,

most episodes only lasting for only one or two days, and that, that diarrhoetic and vomiting episodes often concur simultaneously. They also concluded and that the majority of these patients do not require medical or surgical intervention.

The second questionnaire was developed to assess gastrointestinal reactions to chemotherapeutic treatment. The treatment was in general well tolerated by all dogs based on the reported incidence of gastrointestinal adverse events, as well as on the improved, or static, estimated quality of life and activity level of the dogs. Two-thirds of the treated dogs (67%) experienced different grades of gastrointestinal events following chemotherapeutic treatment. The results from this study may agree with the general knowledge that some dogs may experience adverse events from the gastrointestinal tract after the use of cytostatic medication, as described in other studies (Chavalle *et al*., 2002; Cunha *et al.*, 2017). However, considering the small study population, caution must be applied to its interpretation, as the findings may not be completely reliable. As mentioned above, the results from the studies by Edwards *et al.* (2007), Hubbard *et al.* (2007), and Candellone *et al.* (2020), may support the possibility that the cases of gastrointestinal events found in patients treated with chemotherapy in this study may have been caused by a more or less common gastroenteritis rather than being an adverse event caused by chemotherapeutic medication. This can, however, not be concluded without further research.

Since the findings in questionnaire 1 indicate that the majority of dogs experienced some form of mild disturbance from the gastrointestinal tract, no certain conclusions regarding the causality of chemotherapeutic treatment and the of gastrointestinal adverse events can be drawn at this point. It is not possible to determine whether the gastrointestinal events were caused by a common gastroenteritis, reactions to cytostatic medication, or if they had another aetiology. A larger study population is required for more conclusive results.

5.3 Limitations & sources of error

There are several limitations and sources of error in this study that need to be addressed. Firstly, due to the size of the study populations, the results and the subsequent interpretation of them may not be representative for a larger population. Data and samples from a larger number of patients could show more significant results and correlations. Secondly, the time for return visits after chemotherapeutic treatment is not standardised between patients due to different treatment protocols and owner compliance. This could possibly affect the detectable concentrations of calprotectin and acute changes in the gut microbiota since symptoms and analysable alterations may be most prominent during the first days after treatment. This can

make the comparison between individuals difficult. Since analyses of calprotectin and microbiota could not be performed due to practical constraints, further hypothesises of sources of error in these methods and their possible results will not be included in this report.

Both questionnaires were written to get an overview of the life habits, treatments, well-being, and gastrointestinal disturbances both prior to, and after, chemo-therapeutic treatment. However, these questionnaires included several limitations. The risk of bias and other sources of error is generally high in this type of questionnaire-based research. Some of the questions included in the first part of the questionnaire refer to gastrointestinal events during the last year. Due to this long time frame, owners might not be able to recollect every episode of gastrointestinal disturbance, nor their duration, severity or treatment. Hence, there is a considerable risk of recall bias in questionnaire-based studies. Another important factor to take into consideration is that some questions asked in both questionnaires were subjective and might be left open to interpretation. This means that the intended answers might not be provided, possibly discrediting the results. Additionally, the questions used in the questionnaires are subjective to the owner's opinions and sightings. Gastrointestinal events might go unnoticed depending on whether the dog shows any clinical symptoms, as well as on the attentiveness of the pet owner.

Additionally, the inability to analyse the faecal samples collected in this study makes it difficult to determine whether the methods for collection and further processing of these samples were effective. The collection, handling, storing and processing of these samples are all able to influence the outcome of the analyses. Modifications of the management of the samples might have to be performed after its effectiveness has been reviewed in order to confirm its validity.

5.4 Potential therapeutic value

5.4.1 The potential therapeutic value of faecal calprotectin

The use of faecal calprotectin as a non-invasive biomarker for inflammation in the gastrointestinal tract has been validated in human medicine, mainly for chronic inflammatory states (Smith & Gaya, 2012; Bjarnason, 2017; Pathirana *et al.*, 2017). In small animal veterinary medicine, the use of the protein is currently limited, but several studies have established faecal calprotectin as a potentially useful marker of disease severity in dogs with inflammatory gastrointestinal disease (Grellet *et al.*, 2012; Heilmann, 2015; Otoni *et al.*, 2018). The use in acute inflammatory conditions in animal patients has not been investigated as frequently, but due to calprotectin's presence during both acute and chronic inflammatory states,

calprotectin has the potential to be used in both cases. A favourable aspect of calprotectin as a biomarker for the evaluation of intestinal health is that it is non-invasive, resulting in reduced stress for the patient in comparison to other diagnostic methods. Further studies regarding the clinical utility of faecal calprotectin are warranted to evaluate its usefulness as a predictor of gastrointestinal adverse events following chemotherapy, which could result in improved treatment and quality of life in dogs with cancer.

5.4.2 The potential therapeutic value of the gut microbiota

The normal gut microbiota in canine patients has been mapped in several studies and it has been found that cancer and systemic cancer therapies may induce changes in the normal diversity and composition (Iida *et al.*, 2013; Zitvogel *et al*., 2017). The gut microbiota can also mediate the patient response to chemotherapy through different modulatory effects, and the immunomodulatory effects of some cytostatic agents require a functional microbiome. It can therefore be hypothesised that the differences in the microbial composition in the gastrointestinal tract may be a cause of the different toxicity profiles and responses to chemotherapeutic treatment.

However, research on this topic regarding dogs is scarce, and there are no definitive results on the chemotherapeutic impact on the microbial gut ecosystem and the connections to the development of adverse events in canine patients. Further studies are therefore required to determine the chemotherapeutic effects on gut homeostasis in canine patients, and to determine whether there are any types of microbial compositions that make individuals more or less predisposed to developing adverse events after chemotherapy.

5.5 Conclusion

The questionnaires used in this study could potentially be used to evaluate and find possible influential factors that might affect the development of gastrointestinal toxicity, and to objectively evaluate findings of gastrointestinal toxicity after chemotherapeutic treatment. Many questions used in the questionnaires are extensive and could therefore be relevant for many types of studies reviewing the same, or other related topics. However, to be able to draw accurate and significant conclusions regarding the usability of the questionnaires, the study population must be significantly larger**.** Hence, further investigations regarding their usability must be conducted.

No definitive conclusions can at this point be drawn regarding the use of calprotectin and the gut microbiota as biomarkers for the risk of the development

of gastrointestinal adverse events following chemotherapy. However, it is possible to, at least to some extent, speculate regarding their theoretic usability. The potential of the gut microbiota as an indicator for the risk of suffering from chemotherapy-induced adverse events from the gastrointestinal tract shows promise in the literature due to its' importance for the homeostasis and mediation of different therapeutic responses. Current research also suggest that faecal calprotectin shows potential as a non-invasive biomarker in both human and animal medicine due to its association with chronic and acute inflammation in the gut.

5.6 Further research and studies

This study reviews and studies an essential topic for future research since the effects of adverse events significantly can affect the quality of life of patients during cytostatic treatment. The use of a questionnaire for reviewing risk factors for disease could prove to be a beneficial and non-invasive way of anticipating gastro-intestinal toxicity. In the long run, the possibility of using biomarkers prior to chemotherapeutic treatment could also be favourable to predict which individuals might develop adverse events after cytostatic therapy. Using identified risk factors and biomarkers, the clinician might be able to make more well-founded decisions regarding treatment protocol and optimisation of prophylactic measures. This may also allow the clinician to focus the use of preventative measures on predisposed individuals.

While this study and literature overview provides an insight into the current knowledge and potential use of biomarkers, further studies regarding calprotectin, the gut microbiota and chemotherapy must be performed. The faecal samples collected within the time frame for this thesis could later be used to investigate the connection between chemotherapeutic treatment and gastrointestinal toxicity by studying the microbial composition in the gastrointestinal tract prior to and after cytostatic treatment. This may give an insight into how homeostasis in the gastrointestinal tract may be affected by cytotoxic changes. A mapping of the relative proportions of certain microorganisms in the gut microbiota may be useful to determine the risk of developing dysbiosis and/or adverse events of different severities. Another objective, in the prolongation of this study, could be to also investigate the use of faecal calprotectin and its association with the development of adverse events in the gastrointestinal tract following chemotherapy. Other influential factors such as the type of cancer and its location as well as the effects of comorbidities or comedication should also be investigated in future studies due to their potential impact on the therapeutic effect of cytostatic medication, the inflammation in the gut and effect on the gut ecosystem.

Further research and studies with a larger study population are required to identify risk factors and evaluate the usability of the questionnaires. Additionally, studies reviewing the relationship between cytostatic treatment, the intestinal microbiota and inflammatory markers in faeces have to be performed in order to determine their actual clinical value.

References

Aarnoutse, R., Ziemons, J., Penders, J., Rensen, S.S., de Vos-Geelen, J. & Smidt, M.L. (2019). The clinical link between human intestinal microbiota and systemic cancer therapy. *International Journal of Molecular Science,* 20, 4145.

Abbexa (2022). Dog Calprotectin ELISA Kit. https://www.abbexa.com/calprotectin-calpro-elisa-kit

Abt, M.C., Osborne, L.C., Monticelli, L.A., Doering, T.A., Alenghat, T., Sonnenberg, G.F., Paley, M.A., Antenus, M., Williams, K.L., Erikson, J., Wherry, E.J. & Artis, D. (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*. 37(1), 158-70. doi: 10.1016/j.immuni.2012.04.011. Epub 2012 Jun 14. PMID: 22705104; PMCID: PMC3679670.

Adams, V.J., Evans, K.M., Sampson, J., & Wood, J.L. (2010). Methods and mortality results of a health survey of purebred dogs in the UK. *The Journal of Small Animal Practice*, 51(10), 512–524. https://doi.org/10.1111/j.1748-5827.2010.00974.x

Alexander, J.L., Wilson, I.D., Teare, J., Marchesi, J.R., Nicholson, J.K. & Kinross, J.M. (2017). Gut microbiota modulation of chemotherapy efficacy and toxicity. *Nature Reviews Gastroenterology & Hepatology*, 14(6), 356–365. Available at: https://dx.doi.org/10.1038/nrgastro.2017.20.

AlShawaqfeh, M.K., Wajid, B., Minamoto, Y., Markel, M.., Lidbury, J.A., Steiner, J.M., Serpedin, E. & Suchodolski, J.S. (2017). A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. *FEMS Microbiology Ecology*, 93(11), 10.1093/femsec/fix136. https://doi.org/10.1093/femsec/fix136

Backlund, A. (2021). Cytostatikabiverkningar hos hund och katt – en litteraturstudie. *Svensk Veterinärtidning*, 73, 22-26, https://www.svenskveterinartidning.se/SVT_2107_inlaga_webb.pdf [2022-09-15]

Bjarnason, I. (2017). The use of fecal calprotectin in inflammatory bowel disease. *Gastroenterology & Hepatology*, 13(1), 53-56. https://doi.org/https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5390326/

Berg, G., Rybakova, D., Fischer, D. *et al.* (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome*, 8, 103. https://doi.org/10.1186/s40168-020-00875-0

Boussac, M. & Garin, J. (2000) Calcium-dependent secretion in human neutrophils: a proteomic approach. *Electrophoresis*, 21, 665-672.

https://doi.org/10.1002/(SICI)1522-2683(20000201)21:3<665::AID-ELPS665>3.0.CO;2-U

Bronson, R.T. (1982). Variation in age at death of dogs of different sexes and breeds. *American Journal of Veterinary Research*, 43, 2057–2059.

Candellone, A., Cerquetella, M., Girolami, F., Badino, P. & Odore, R. (2020). Acute diarrhea in dogs: current management and potential role of dietary polyphenols supplementation. *Antioxidants*, 9(8). https://doi.org/10.3390/antiox9080725

Chavalle, T., Chamel, G., Denoeux, P., Lajoine, M., Sayag, D., Berny, P. & Ponce, F. (2022). Are severe adverse events commonly observed in dogs during cancer chemotherapy? A retrospective study on 155 dogs. *Veterinary and Comparative Oncology*, 20(2), 393–403. https:///doi.org/10.1111/vco.12782

Coussens, L.M. & Werb, Z. (2002). Inflammation and cancer. *Nature*, 420(6917), 860-867. Doi: https://doi.org/10.1038/nature01322

Cunha, S.C.S., Silva, F.B.F., Corgozinho, K.B., Silva, K.V.G. & Ferreira, A.M.R. (2017). Adverse effects of chemotherapy in dogs. *World Veterinary Journal*, 7(3), 74-82. Doi: http://dx.doi.org/10.5455/wvj.20170896

Daillère, R., Vétizou, M., Waldschmitt, N., *et al.* (2016). Enterococcus hirae and Barnesiella intestinihominis facilitate cyclophosphamide-induced therapeutic immunomodulatory effects. *Immunity*, 45(4), 931-943. https://doi:10.1016/j.immuni.2016.09.009

Desselberger, U. (2018). The mammalian intestinal microbiome: composition, interaction with the immune system, significance for vaccine efficacy, and potential for disease therapy. *Pathogens*, (Basel, Switzerland), 7(3) 57. https://doi.org/10.3390/pathogens7030057

Dobson, J.M. (2013). Breed-predispositions to cancer in pedigree dogs. *ISRN Veterinary Science*, 1–23. https://dx.doi.org/10.1155/2013/941275.

Dupré, J.O. & O'Malley, M.A. (2009). Varieties of living things: life at the intersection of lineage and metabolism. In: Normandin, S. & Wolfe, C. (eds.). *Vitalism and the Scientific Image in Post-Enlightenment Life Science*; *History, Philosophy and Theory of the Life Sciences.* Dordrecht: Springer; p. 1800–2010. http://doi.org/10.3998/ptb.6959004.0001.003

Edwards, D.S., Henley, W.E., Ely, E.R., & Wood, J.L. (2004). Vaccination and ill-health in dogs: a lack of temporal association and evidence of equivalence. *Vaccine*, 22(25-26), 3270–3273. https://doi.org/10.1016/j.vaccine.2004.03.038

Elting, L.S., Cooksley, C., Chambers, M., Cantor, S.B., Manzullo, E. & Rubenstein, E.B. (2003). *The burdens of cancer therapy. Clinical and economic outcomes of chemotherapy-induced mucositis. Cancer,* 98, 1531– 1539. https://doi.org/10.1002/cncr.11671

Enderle, L.L., Köller, G., & Heilmann, R.M. (2022). Verification of the fCAL turbo immunoturbidimetric assay for measurement of the fecal calprotectin concentration in dogs and cats. *Journal of Veterinary Diagnostic Investigation: Official Publication of*

the American Association of Veterinary Laboratory Diagnosticians, Inc., 34(5), 813-824. https://doi.org/10.1177/10406387221114031

Epiphanio, T.M.F. & Santos, A. (2021). Small animals gut microbiome and its relationship with cancer. In Rutland, C (ed.), *Canine Genetics, Health and Medicine*, IntechOpen, London. 10.5772/intechopen.95780.

Fagerhol, M.K., Dale, I. & Andersson, T. (1980). A radioimmunoassay for a granulocyte protein as a marker in studies on the turnover of such cells. *Bulletin Europeen de Physiopathologie Respiratoire,* 16 (supplement), 273–282. https://doi.org/10.1016/b978-0-08-027379-2.50028-4

Farese, J.P. & Withrow., J.S., (2013). Chapter 10 - Surgical oncology. In: Withrow, S.J., Vail, D.M. & Page, R.L. (eds.) *Withrow and MacEwen's Small Animal Clinical Oncology* (Fifth Edition). Saint Louis: W.B. Saunders. 157–179. https://doi.org/10.1016/B978-1-4377-2362-5.00011-6

Fengming, Y. & Jianbing, W. (2014). Biomarkers of inflammatory bowel disease. *Disease Markers,* 710915. https://doi.org/10.1155/2014/710915

Fraher, M.H., O'Toole, P.W. & Quigley, E.M. (2012) Techniques used to characterize the gut microbiota: a guide for the clinician. *Nature Reviews. Gastroenterology & Hepatology,* 9, 312–322.

German, A.J., Day, M.J., Ruaux C.G., Steiner, J.M., Williams, D.A. & Hall E.J. (2003). Comparison of direct and indirect tests for small intestinal bacterial overgrowth and antibiotic-responsive diarrhea in dogs. *Journal of Veterinary Internal Medicine* 17(1), 33–43. https://doi.org/10.1892/0891-6640(2003)017<0033:codait>2.3.co;2

Gibson, R.J., & Keefe, D.M. (2006). Cancer chemotherapy-induced diarrhoea and constipation: mechanisms of damage and prevention strategies. *Supportive Care in Cancer : Official Journal of the Multinational Association of Supportive Care in Cancer,* 14(9), 890–900. https://doi.org/10.1007/s00520-006-0040-y

Giuffrida, M.A., Farrar, J.T,. Brown, D.C. *et al.* (2017). Psychometric properties of the Canine Symptom Assessment Scale, a multidimensional owner-reported questionnaire instrument for assessment of physical symptoms in dogs with solid tumors. *Journal of the American Veterinary Medical Association,* 251, 1405-1414. https://doi.org/10.2460/javma.251.12.1405

Grellet, A., Heilmann, R.M., Lecoindre, P., Feugier, A., Day, M.J., Peeters, D., Freiche, V., Hernandez, J., Grandjean, D., Suchodolski, J.S. & Steiner, J.M. (2013). Fecal calprotectin concentrations in adult dogs with chronic diarrhea. *American Journal of Veterinary Research*, 74, 706-711. https://doi.org/10.2460/ajvr.74.5.706

Heilmann, R.M., Suchodolski, J.S., & Steiner, J.M. (2008). Development and analytic validation of a radioimmunoassay for the quantification of canine calprotectin in serum and feces from dogs. *American Journal of Veterinary Research,* 69(7), 845–853. https://doi.org/10.2460/ajvr.69.7.845

Heilmann, R. M., & Allenspach, K. (2017). Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies-brief review. *Journal of Veterinary Diagnostic Investigation : Official Publication of the American Association*

of Veterinary Laboratory Diagnosticians, Inc., 29(6), 781–787. https://doi.org/10.1177/1040638717728545

Heilmann, R. M., Berghoff, N., Mansell, J., Grützner, N., Parnell, N. K., Gurtner, C., Suchodolski, J. S., & Steiner, J. M. (2018). Association of fecal calprotectin concentrations with disease severity, response to treatment, and other biomarkers in dogs with chronic inflammatory enteropathies. *Journal of Veterinary Internal Medicine,* 32(2), 679-692. https://doi.org/10.1111/jvim.15065

Heilmann, R.M. (2015.) *Evaluation of Canine S100A12 and sRAGE as NovelDisease Markers in Dogs with Inflammatory Bowel Disease*. Diss. College Station, TX: Texas A&M University; doi: 10.1111/jvim.15761.

Herstad, K.M.V., Moen, A.E.F., Gaby, J.C., Moe, L. & Skancke, E. (2018). Characterization of the fecal and mucosa-associated microbiota in dogs with colorectal epithelial tumors. *PLoS ONE,* 13(5), e0198342. Available at: https://dx.doi.org/10.1371/journal.pone.0198342.

Hesketh, P.J. (2008). Chemotherapy-induced nausea and vomiting. *New England Journal of Medicine,* 358(23), 2482–2494. https://doi.org/10.1056/NEJMra0706547

Honneffer, J.B., Minamoto, Y. & Suchodolski, J.S. (2014). Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. *World Journal of Gastroenterology,* 20(44), 16489-16497. https://doi.org/10.3748/wjg.v20.i44.16489

Hubbard, K., Skelly, B.J., McKelvie, J. & Wood, J.L.N. (2007). Risk of vomiting and diarrhoea in dogs. *Veterinary Record,* 161, 755-757. https://doi.org/10.1136/vr.161.22.755

Iida, N., Dzutsev, A., Stewart, C.A., Smith, L., Bouladoux, N., Weingarten, R.A., Molina, D.A., Salcedo, R., Back, T., Cramer, S., Dai, R.M., Kiu, H., Cardone, M., Naik, S., Patri, A.K., Wang, E., Marincola, F.M., Frank, K.M., Belkaid, Y., Trinchieri, G. & Goldszmid, R.S. (2013). Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science* (New York, N.Y.), 342(6161), 967–970. https://doi.org/10.1126/science.1240527

Jandhyala, S.M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Reddy, D.N. (2015). Role of the normal gut microbiota. *World Journal of Gastroenterology : WJG,* 21(29), 8787-8803. https://doi.org/10.3748/wjg.v21.i29.8787

Jia, W., Li, H., Zhao, L. & Nicholson, J.K. (2008). Gut microbiota: a potential new territory for drug targeting. *Nature Reviews. Drug Discovery,* 7(2), 123-129, https://doi.org/10.1038/nrd2505

Johnston K.L. (1999). Small intestinal bacterial overgrowth. *The Veterinary Clinics of North America. Small Animal Practice,* 29(2), 523–550.

Khan, A.A., Shrivastava, A., & Khurshid, M. (2012). Normal to cancer microbiome transformation and its implication in cancer diagnosis. *Biochimica et Biophysica Acta,* 1826(2), 331–337. https://doi.org/10.1016/j.bbcan.2012.05.005

LeBlanc, A.K., Atherton, M., Bentley, R.T., Boudreau, C.E., Burton, J.H., Curran, K.M., Dow, S., Giuffrida, M.A., Kellihan, H.B., Mason, N.J., Oblak, M., Selmic, L.E., Selting, K.A., Singh, A., Tjostheim, S., Vail, D.M., Weishaar, K.M., Berger, E.P.,

Rossmeisl, J.H. & Mazcko, C. (2021). Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE v2) following investigational therapy in dogs and cats. *Veterinary Comparative Oncology*, 19(2), 311-352. doi: 10.1111/vco.12677. Epub 2021 Feb 18. PMID: 33427378; PMCID: PMC8248125.

Lehouritis, P., Cummins, J., Stanton, M., Murphy, C.T., McCarthy, F.O., Reid, G., Urbaniak, C., Byrne, W.L. & Tangney, M. (2015). Local bacteria affect the efficacy of chemotherapeutic drugs. *Scientific Reports*, 5(1): 14554. https://doi.org/10.1038/srep14554

Logan, R.M., Gibson, R.J., Bowen, J.M., Stringer, A.M., Sonis, S.T. & Keefe, D.M. (2008). Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. *Cancer Chemotherapy and Pharmacology*, 62(1), 33–41. https://doi.org/10.1007/s00280-007-0570-0

Marchesi, J.R. & Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome*, 3, 31. https://doi.org/10.1186/s40168-015-0094-5

Mentula, S., Harmoinen, J., Heikkila, M., Westermarck, E., Rautio, M., Huovinen, P. & Kononen, E. (2005). Comparison between cultured small-intestinal and fecal microbiotas in Beagle dogs. *Applied and Environmental Microbiology*, 71(8), 4169–4175. https://doi.org/10.1128/AEM.71.8.4169-4175.2005

Middelbos, I.S., Vester Boler, B.M., Qu, A., White, B.A. & Swanson, K.S. (2010). Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. *PLoS ONE*, 5(3). https://doi.org/10.1371/journal.pone.0009768

Minamoto, Y., Otoni, C.C., Steelman, S.M., Büyükleblebici, O., Steiner, J.M., Jergens, A.E. & Suchodolski, J.S. (2015). Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. *Gut Microbes*, 6(1), 33-47. https://doi.org/10.1080/19490976.2014.997612

Omori, M., Maeda, S., Igarashi, H., Ohno, K., Sakai, K., Yonezawa, T., Horigome, A., Odamaki, T. & Matsuki, N. (2017). Fecal microbiome in dogs with inflammatory bowel disease and intestinal lymphoma. *The Journal of Veterinary Medical Science*, 79(11), 1840–1847. https://doi.org/10.1292/jvms.17-0045

Otoni, C.C., Heilmann, R.M., Sainz, A., Ackermann, M.R., Suchodolski, J.S., Steiner, J.M. & Jergens, A.E. (2018). *Serologic and fecal markers to predict response to induction therapy in dogs with idiopathic inflammatory bowel disease*. *Journal of Veterinary Internal Medicine*, 32(3), 999-1008. https://doi.org/10.1111/jvim.15123

Overmann, J., Abt, B. & Sikorski J. (2017). Present and future of culturing bacteria. *Annual Review of Microbiology*, 71, 711–730. https://doi.org/10.1146/annurev-micro-090816-093449

Pagano, J.S., Blaser, M., Buendia, M.A., Damania, B., Khalili, K., Raab-Traub, N. & Roizman, B. (2004). Infectious agents and cancer: criteria for a causal relation. *Seminars in Cancer Biology*, 14(6), 453–471. https://doi.org/10.1016/j.semcancer.2004.06.009

Pang, L.Y., & Argyle, D.J. (2016). Veterinary oncology: Biology, big data and precision medicine. *The Veterinary Journal*, 213, 38–45. https://dx.doi.org/10.1016/j.tvjl.2016.03.009.

Pathirana, W.G., Chubb, S.P., Gillett, M.J. & Vasikaran, S.D. (2018). Faecal calprotectin. *The Clinical Biochemist Reviews*, 39(3), 77-90. https://doi.org/https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6370282/

Peek, R.M. & Blaser, M.J. (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nature Reviews. Cancer*, 2, 28–37. https://doi.org/10.1038/nrc703

Petersen, C. & Round, J.L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, 16(7), 1024-1033. https://doi.org/10.1111/cmi.12308

Pilla, R. & Suchodolski, J.S. (2019). The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. *Frontiers in Veterinary Science*, 6, 498. https://doi.org/10.3389/fvets.2019.00498

Pinello, K., Amorim, I., Pires, I., Canadas-Sopillausa, A., Catarino, J., Faísca, P., Branco, S., Peleteiro, M.C., Silva, D., Severo, M. & Niza-Ribeiro, J. (2022). Vet-OncoNet: Malignancy analysis of neoplasms in dogs and cats, *Veterinary Sciences* 9(10), 535. https://doi.org/10.3390/vetsci9100535

Polk, D.B., & Peek, R.M.Jr. (2010). Helicobacter pylori: gastric cancer and beyond. *Nature Reviews. Cancer*, 10(6), 403–414. https://doi.org/10.1038/nrc2857

Proschowsky, H.F., Rugbjerg, H. & Ersbøll, A.K. (2003). Mortality of purebred and mixed-breed dogs in Denmark. *Preventive Veterinary Medicine*, 58(1–2, 63-74. ISSN 0167-5877, https://doi.org/10.1016/S0167-5877(03)00010-2.

Regan, D., Guth, A., Coy, J. & Dow, S. (2016). Cancer immunotherapy in veterinary medicine: Current options and new developments. *Veterinary Journal* (London, England : 1997), 207, 20–28. https://doi.org/10.1016/j.tvjl.2015.10.008

Reif, J.S. (2007). Chapter 4 - The epidemiology and incidence of cancer, In: Withrow, S.J. & Vail, D.M. (eds.) *Withrow & MacEwen's Small Animal Clinical Oncology* (Fourth Edition), W.B. Saunders, Pages 68-76, ISBN 9780721605586, https://doi.org/10.1016/B978-072160558-6.50007-1

Richardson, G. & Dobish, R. (2007). Chemotherapy induced diarrhea. *Journal of Oncology Pharmacy Practice : official publication of the International Society of Oncology Pharmacy Practitioners*, 13(4), 181–198. https://doi.org/10.1177/1078155207077335

Rivera-Chavez, F., Lopez, C.A. & Baumler, A.J. (2017). Oxygen as a driver of gut dysbiosis. *Free Radical Biology and Medicine*, 105, 93–101. https://doi.org/10.1016/j.freeradbiomed.2016.09.022

Rosadi, F., Fiorentini, C. & Fabbri, A. (2016). Bacterial protein toxins in human cancers. *Pathogens and Disease.* 74(1):ftv105. https://doi.org/10.1093/femspd/ftv105

Scheline, R.R. (1968). Drug metabolism by intestinal microorganisms. *Journal of Pharmaceutical Sciences*, 57, 2021-2037. https://doi.org/10.1002/jps.2600571202

Schwabe, R.F. & Jobin, C. (2013). The microbiome and cancer. *Nature Reviews. Cancer*. 13(11), 800-812. https://doi.org/10.1038/nrc3610

Simpson, J.M., Martineau B., Jones W.E., Ballam J.M. & Mackie R.I. (2002). Characterization of fecal bacterial populations in canines: Effects of age, breed and dietary fiber. *Microbial Ecology*, 44, 86–197. https://doi.org/10.1007/s00248-002-0001-z

Smith, LA. & Gaya, D.R. (2012). Utility of faecal calprotectin analysis in adult inflammatory bowel disease. *World Journal of Gastroenterology*, 18(46), 6782–6789. https://doi.org/10.3748/wjg.v18.i46.6782

Stein, A., Voigt, W., & Jordan, K. (2010). Chemotherapy-induced diarrhea: pathophysiology, frequency and guideline-based management. *Therapeutic Advances in Medical Oncology*, 2(1), 51-63. https://doi.org/10.1177/1758834009355164

Stríz, I. & Trebichavský, I. (2004). Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiological Research*, 53(3), 245–253.

Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D. & Doré, J. (1999*).* Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Applied and Environmental Microbiology*, 65(11), 4799–4807. https://doi.org/10.1128/AEM.65.11.4799-4807.1999

Suchodolski, J.S. (2011). Companion Animals Symposium: Microbes and gastrointestinal health of dogs and cats. *Journal of Animal Science*, 89(5), 1520–1530. https://doi.org/10.2527/jas.2010-3377

Suchodolski, J.S. (2016). Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *Recent Developments in Veterinary Diagnostics*, Special Issue, 215, 30–37. https://doi.org/10.1016/j.tvjl.2016.04.011

Suchodolski, J.S., Markel, M.E., Garcia-Mazcorro, J.F., Unterer, S., Heilmann, R.M., Dowd, S.E., Kachroo, P., Ivanov, I., Minamoto, Y., Dillman, E.M., Steiner, J.M., Cook, A.K. & Toresson, L. (2012). The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS ONE*, 7(12), e51907. https://dx.doi.org/10.1371/journal.pone.0051907

Suchodolski, J.S., Camacho, J. & Steiner, J.M. (2008a). Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. *FEMS Microbiology Ecology*, 66(3), 567-578. https://doi.org/10.1111/j.1574-6941.2008.00521.x

Suchodolski J.S., Morris E.K., Allenspach K., Jergens A.E., Harmoinen J.A., Westermarck E. & Steiner J.M. (2008b). Prevalence and identification of fungal DNA in the small intestine of healthy dogs and dogs with chronic enteropathies. *Veterinary Microbiology*, 132, 379–388. https://doi.org/10.1016/j.vetmic.2008.05.017

Swanson, K.S., Dowd, S.E., Suchodolski, J.S., Middelbos, I.S., Vester, B.M., Barry, K.A., Nelson, K.E., Torralba, M., Henrissat, B., Coutinho, P.M., Cann, I.K., White, B.A., & Fahey, G.C.Jr., (2011*).* Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *The ISME Journal*, 5(4), 639–649. https://doi.org/10.1038/ismej.2010.162

Thaiwong, T., Sledge, D.G., Wise, A.G., Olstad, K., Maes, R.K., Kiupel, M. (2018). Malignant transformation of canine oral papillomavirus (CPV1)-associated papillomas in dogs: An emerging concern? *Papillomavirus Research* (Amsterdam, Netherlands), 6, 83–89. https://doi.org/10.1016/j.pvr.2018.10.007

Thamm, D.H. (2019). Canine cancer: strategies in experimental therapeutics. *Frontiers in Oncology*, 9. https://doi.org/10.3389/fonc.2019.01257

Tomiyasu, H., Takahashi, M., Fujino, Y., Ohno, K. & Tsujimoto, H. (2010). Gastrointestinal and hematologic adverse events after administration of vincristine, cyclophosphamide, and doxorubicin in dogs with lymphoma that underwent a combination multidrug chemotherapy protocol. *The Journal of Veterinary Medical Science*, 72(11), 1391–1397. https://doi.org/10.1292/jvms.10-0176

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. & Gordon, J.I. (2007). The human microbiome project. *Nature*, 449(7164), 804-810. https://doi.org/10.1038/nature06244

Vail, D.M. (2009). Supporting the veterinary cancer patient on chemotherapy: neutropenia and gastrointestinal toxicity. *Topics in Companion Animal Medicine*, 24(3), 122–129. https://doi.org/10.1053/j.tcam.2009.02.004

Vazquez-Baeza, Y., Hyde, E R., Suchodolski, J.S. & Knight, R. (2016). Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. *Nature Microbiology*, 1, 16177. https://doi.org/10.1038/nmicrobiol.2016.177

Veterinary Cancer Society (2022). *What are the most common types of cancers in dogs? How many dogs typically get cancer?*. http://vetcancersociety.org/pet-owners/faqs, [2022-10-22]

VCOG (2004). Veterinary co-operative oncology group common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0, *Veterinary and Comparative Oncology*, 2, 194–213.

VCOG (2016). Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and Comparative Oncology*, 14(4), 417–446. https://doi.org/10.1111/vco.283

Viaud, S., Saccheri, F., Mignot, G., Yamazaki, T., Daillère, R., Hannani, D., Enot, D.P., Pfirschke, C., Engblom, C., Pittet, M.J., Schlitzer, A., Ginhoux, F., Apetoh, L., Chachaty, E., Woerther, L., Eberl, G., Bérard, M., Ecobichon, C., Clermont, D., . . . & Zitvogel, L. (2013). The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* (New York, N.Y.), 342(6161), 971. https://doi.org/10.1126/science.1240537

Vich Vila, A., Imhann, F., Collij, V., Jankipersadsing, S.A., Gurry, T., Mujagic, Z., Kurilshikov, A., Bonder, M.J., Jiang, X., Tigchelaar, E.F., Dekens, J., Peters, V., Voskuil, M.D., Visschedijk, M.C., van Dullemen, H.M., Keszthelyi, D., Swertz, M. A., Franke, L., Alberts, R., Festen, E.A.M., … & Weersma, R.K. (2018). Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. *Science Translational Medicine*, 10(472), eaap8914. https://doi.org/10.1126/scitranslmed.aap8914

Wang, S.L., Lee, J.J. & Liao, A.T. (2015). Chemotherapy-induced neutropenia is associated with prolonged remission duration and survival time in canine lymphoma. *Veterinary Journal* (London, England: 1997), 205(1), 69–73. https://doi.org/10.1016/j.tvjl.2015.04.032

Whipps, J., Lewis, K. & Cooke, R. (1988). Mycoparasitism and plant disease control. In: Burge, M. (ed.), *Fungi in Biological Control Systems*. Manchester University Press..p. 161-187.

White, B.A. (2011). Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *The ISME Journal*, 5(4), 639-649. https://doi.org/10.1038/ismej.2010.162

Wilson, I.D. & Nicholson, J.K. (2017). Gut microbiome interactions with drug metabolism, efficacy and toxicity. *Translational Research : The Journal of Laboratory and Clinical Medicine*, 179, 204. https://doi.org/10.1016/j.trsl.2016.08.002

Wolfesberger, B., Tonar, Z., Fuchs-Baumgartinger, A., Walter, I., Skalicky, M., Witter, K., Thalhammer, J. G., Pagitz, M., & Kleiter, M. (2012). Angiogenic markers in canine lymphoma tissues do not predict survival times in chemotherapy treated dogs. *Research in Veterinary Science*, 92(3), 444–450. https://doi.org/10.1016/j.rvsc.2011.04.018

Young, V.B. (2017). The role of the microbiome in human health and disease: an introduction for clinicians. *BMJ*, 356, j831. https://doi.org/10.1136/bmj.j831

Yui, S., Nakatani, Y. & Mikami, M. (2003). Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *Biological and Pharmaceutical Bulletin* 26(6): 753–760. https://doi.org/10.1248/bpb.26.753.

Popular science summary

Cancer and chemotherapy

Cancer diseases are important and common in dogs. There are several treatment options for cancer and the choice of therapy is made based on several aspects of the patient and the cancer type, including but not limited to: the age of the dog, other diseases, the type of cancer as well as the economic capabilities and will of the owner.

Chemotherapy is a type of treatment suitable for some cancer types and certain individuals. This treatment affects rapidly dividing cells to shrink, stop the growth of, or destroy cancer cells. However, since the medication is unable to differentiate between cancer cells and rapidly dividing normal cells, there is a risk that the patient may suffer from side effects, also called adverse events. Rapidly dividing cells can be found in many parts of the body but are mainly found in the gastrointestinal tract and the bone marrow. The most common adverse events after chemotherapy are seen from the gastrointestinal tract, with common symptoms such as nausea, vomiting and diarrhoea. These adverse events may affect the dogs' well-being and quality of life and may also affect the dog owner.

The aim of the study

To improve the quality of life of dogs undergoing chemotherapy, it is important to understand the connections between chemotherapeutic treatment and gastrointestinal adverse events. The possibility of predicting which animals may be at risk for developing gastrointestinal adverse events, and the reason for this, could prove to be of value for veterinarians and other clinicians in order to optimise treatment protocols and reduce the risk of the development of gastrointestinal adverse events.

The ambition of this study is to understand the reason why chemotherapy-induced side effects affect some dogs more than others and why the side effects are more or less severe in different patients. The aim of this study is to find connections between chemotherapy and the development of gastrointestinal side effects through the use of two questionnaires as well as through a literature review regarding potential

biomarkers for the risk of developing chemotherapy-induced gastrointestinal side effects. The first questionnaire has the aim of finding potential influential factors in the everyday life or diet of dogs with cancer that might lead to the development of adverse events from the gut. The second questionnaire is focused on gastrointestinal adverse events, well-being and quality of life in canine cancer patients after chemotherapeutic treatment. In the long run, it could also be possible to use certain markers of disease in combination with these questionnaires to find which animals are at higher risk for the development of gastrointestinal adverse events.

Potential markers of disease

The gut microbiota includes the system of microorganisms found in the gastrointestinal tract. Bacteria are the most common type of microorganism, but other microbes such as fungi, archaea, protozoa, and viruses can also be found. Different types of microorganisms have different functions in the body, and they are collectively involved in maintaining good host health through different spectrums of activity. If the gut microbiota is altered in any way, for example, due to medication, there is a risk that the favourable bacteria die while bacteria with the ability to cause disease might grow excessively. This event of a disruption in the normal gut microbiota is called dysbiosis.

Another way of possibly understanding the effects of chemotherapy on the gastrointestinal tract is using inflammatory markers, often called biomarkers. One possible biomarker for inflammation in the canine gut is faecal calprotectin, a protein routinely used in human medicine for chronic conditions such as inflammatory bowel disease. The use of this biomarker is not common in veterinary medicine, but studies on inflammatory conditions in the gut of dogs have shown that the use of calprotectin could be promising for evaluating inflammatory diseases and events in the gastrointestinal tract**.**

Material and Methods

Participants for this study were found at the University Animal Hospital (UDS) in Uppsala, Sweden. A questionnaire was used to get an overview of possible factors in the diet and day-to-day life of dogs with cancer that might have the possibility to influence their response to chemotherapy. The owners of the dogs treated with chemotherapy also answered a second questionnaire focused on how well the dog responded to the anti-cancer treatment and on if the dog had experienced any gastrointestinal adverse events.

Results and Discussion

To find a study population, the owners were contacted either through email or text messages sent from the University Animal Hospital. An interesting finding was that

only 18% of the owners contacted through email chose to enrol in the study, while more than 50% of the owners contacted through text messages did the same. This suggests that text messages might be a more effective channel to find a study population. A total of eight dogs with cancer were included in the first part of the study, and of these patients, three patients continued with chemotherapeutic treatment and continued their participation in the study.

The results from the first questionnaire showed that the majority of dogs in this study (75%) had experienced either vomiting or diarrhoea during the last year, and that bit more than one-third of the patients had had episodes of both vomiting and diarrhoea during the same period. The second questionnaire found that two-thirds of the chemotherapy-treated dogs had experienced different grades of gastrointestinal adverse events after their treatment. This finding was similar to what has been described in other studies, however, due to the small study population of three individuals, these results are not completely reliable. Since the absolute majority of patients that answered questionnaire 1 had experienced vomiting or diarrhoea, it is not possible to conclude that the only reason for the gastrointestinal disturbances in the patients treated with chemotherapy is related to their treatment.

The study had several imitations. Firstly, one of the greatest limitations of this study was the small study population. Due to this, no significant conclusions could be drawn. A larger study population is essential to be able to draw any conclusions. Secondly, some of the questions asked in the questionnaire were regarding vomiting and diarrhoea during the last year. It might be difficult for the owner to remember the events and duration of the gastrointestinal disturbances during such a long time, and therefore, there is a risk of wrongfully answered questions. Thirdly, some of the questions asked in both the first and second questionnaires rely on the owner's options and sightings of gastrointestinal events. Therefore, such events might go unnoticed, and the frequency of these events can be underestimated, affecting the result of this questionnaire.

Conclusions

The questionnaires used in this study could potentially be used to evaluate and find possible influential factors or risk factors that might affect the development of gastrointestinal adverse events after chemotherapy. They might also be used to objectively evaluate the findings and severity of these adverse events. Since many of the questions used in these questionnaires are wide-ranging, they might be able to be relevant for other studies reviewing the same or similar topics. However, to be able to draw accurate conclusions regarding the usability of the questionnaires, the study population must be significantly larger.

No conclusions can at this moment be drawn regarding the use of biomarkers for predicting which animals may be at risk for developing gastrointestinal adverse events after chemotherapy. However, their use show promise in the available literature.

Further Research and Studies

This study and literature review gives an insight into the current knowledge and the possible use of questionnaires and biomarkers, but further and more extensive studies must be conducted for a wider understanding of the relationship between chemotherapy, inflammatory markers in stool and the gut microbiota. Understanding gastrointestinal adverse events, and how to predict and prevent them, is of great importance for the well-being and quality of life of dogs that are or will be treated with chemotherapy.

Acknowledgements

I would like to thank my supervisors and teachers Henrik Rönnberg and Sara Saellström for great guidance, support, patience and positivity during the process of writing my master's thesis. I would also like to thank my examiner Ingrid Ljungvall for the helpful feedback I received.

I am also very grateful for the support of my family and friends, both for their never-ending help during this thesis and for their enthusiasm during my studies to become a veterinarian. Thank you for your valuable support, encouragements and for sending me cute animal pictures when I needed motivation.

Appendix 1

Questionnaire 1

Datum:

ENKÄT 1

Min hunds matvanor

Ringa in de alternativ som passar för din hund.

1. Min hund äter i huvudsak:
   a) Kommersiellt hundfoder på burk (blötfoder)
      - Vilket? _____
   b) Kommersiellt torrfoder för hund
      - Vilket? _____
   c) Rått, färskt kött utan ben
   d) Rått, färskt kött med ben
   e) Tillagat färskt kött utan ben
   f) Tillagat färskt kött med ben
   g) Vegetarisk diet
   h) Matrester

2. Förutom sina måltider, får min hund även äta/gnaga på följande. Ange endast om hunden får det varje vecka eller oftare:
   a) Tuggben
   b) Märgben
   c) Godbitar (kommersiellt tillverkat hundgodis, t ex Frolic)
   d) Tuggbitar för ökad munhälsa (t ex Dentastix)
   e) Matrester
   f) Leksaker fyllda med godbitar
   g) Annat: _____

3. Utöver måltider och annan mat/godis, får din hund någon form av fodertillskott? *(t.ex. probiotika, prebiotika, fiskolja, CBD-olja, grönläppad mussla).*
   a) Ja
   b) Nej

Om ja, vilket/vilka tillskott och hur ofta? _____
_____
_____

Min hunds mående

Kräkning

Ringa in de alternativ som passar in för din hund:

1. Har din hund haft kräkningar under det senaste året?
    a. Ja
    b. Nej

Om du svarat "Ja" på ovanstående fråga (fråga 1), svara då även på följande frågor om kräkning. Ringa in de alternativ som passar in för din hund och/eller skriv i fritext på linjen.

2. Har din hund haft flera episoder av kräkningar vid olika tillfällen under det senaste året?
    a. Ja
    b. Nej

3. Hur länge kräktes din hund vid den senaste episoden av kräkning (en eller flera tillfällen per dag)?
    a. 1 dag
    b. 2 dagar
    c. 3 dagar eller fler

4. Har din hund behandlats för kräkning under det senaste året?
    a. Ja
    b. Nej

5. Om du svarat ja på fråga 4: vilken/vilka typ(er) av behandling(ar) genomförs/genomfördes?
    a. Vila
    b. Foderbyte
    c. Medicinering
    d. Inskrivning på djursjukhus för behandling
    e. Operation
    f. Annat: _____

Diarré

Ringa in alla alternativ som passar in.

1. Har din hund haft diarré under det senaste året?
    a. Ja
    b. Nej

Om du svarat "Ja" på ovanstående fråga (fråga 1), svara då även på följande frågor om diarré. Ringa in de alternativ som passar in för din hund och/eller skriv i fritext på linjen.

2. Har din hund haft flera episoder av diarréer vid olika tillfällen under det senaste året?
    a. Ja
    b. Nej

3. Hur länge hade din hund diarré vid den senaste episoden (en eller flera tillfällen per dag)?
    a. 1 dag
    b. 2 dagar
    c. 3 dagar eller fler

4. Har din hund behandlats för diarré under det senaste året?
    a. Ja
    b. Nej

5. Om du svarat ja på fråga 4: Vilken/vilka typ(er) av behandling(ar) genomförs/genomfördes?
    a. Vila
    b. Foderbyte
    c. Medicinering
    d. Inskrivning på djursjukhus för behandling
    e. Operation
    f. Annat: _____

Sjukdomar & medicinering

Ringa in alla alternativ som passar in.

1. Har din hund någon underliggande sjukdom *(utöver cancer)*?
    a. Ja
    b. Nej

Om du svarat "Ja" på ovanstående fråga (fråga 1), svara då även på följande frågor om din hunds sjukdomar och medicinering. Ringa in de alternativ som passar in för din hund och/eller skriv i fritext på linjen.

2. Vilken/vilka sjukdom/-ar har din hund? *(exempelvis allergi, IBD, njur- eller leversjukdom, hormonell sjukdom, hudsjukdom, hjärtsjukdom):*

_____
_____
_____

3. Hur länge har din hund haft denna sjukdom/dessa sjukdomar?
    a) Mindre än 6 månader
    b) Mer än 6 månader, mindre än 1 år
    c) Mer än 1 år

4. Står din hund på någon behandling mot denna sjukdom/dessa sjukdomar?
    a) Ja
    b) Nej

5. Om du svarat ja på fråga 4, vilken behandling står din hund på?
    a) Specialfoder, isåfall vilket/vilka?:

    _____
    _____

    b) Tillskott, isåfall vilket/vilka?:

    _____
    _____

5. (fortsättning)

c) Medicinering, isåfall vilket/vilka?

_____
_____
_____
_____

d) Annat:

_____
_____

6. Har din hund behandlats med antibiotika under de senaste 3 månaderna?
   a. Ja
   b. Nej

7. Behandlas din hund med några andra mediciner i hemmet utöver de som nämnts ovan (fråga 5 & 6) under det senaste halvåret?
   a. Ja
   b. Nej

8. Om du svarat ja på fråga 7, vilken/vilka mediciner behandlas din hund med? *(Inkludera inte de du redan har nämnt i fråga 5)*

_____
_____
_____

Hur bedömer du din hunds aktivitetsnivå just nu?

1. Normal
2. Lindrigt trött
3. Måttligt trött, orkar endast gå upp för avföring och urinering samt för att äta.
4. Mycket trött, måste skrivas in på djursjukhus för att äta och få hjälp med urinerings- och avföringsbeteende.

Hur upplever du hundens livskvalitet just nu? *(hundens livssituation och välmående)*

1. Normal livskvalitet
2. Lindrigt nedsatt livskvalitet
3. Kraftigt nedsatt livskvalitet

Tack för ditt deltagande!

Appendix 2

Questionnaire 2

Datum:

ENKÄT 2

Denna enkät besvaras vid första återbesöket efter påbörjad cytostatikabehandling.

Din hunds mående efter cytostatikabehandling

1. Har din hund påbörjat någon ny medicinering sedan cancerbehandlingen påbörjats, utöver föreskriven medicinering i samband med cytostatikabehandlingen? *(t. ex. smärtlindring, behandling mot eventuella biverkningar, antibiotika)*
    a. Ja
    b. Nej

    Om ja, vilket/vilka läkemedel har tillkommit och när började hunden behandlas med det? _____
    _____
    _____
    _____

2. Har din hund oplanerat avslutat någon medicinering sedan cancerbehandlingen påbörjats? (*t.ex. smärtlindring, läkemedel som orsakat biverkningar, annat)*
    a. Ja
    b. Nej

    Om ja, vilken medicinering har avslutats? När och varför avslutades behandlingen?_____
    _____
    _____
    _____

3. Har din hund behövt besöka veterinär eller skrivas in på djursjukhus sedan cancerbehandlingen påbörjats?
    a. Ja
    b. Nej

    Om ja, när och varför? _____
    _____

Nedan följer en lista på möjliga biverkningar från mag-tarmkanalen efter cytostatikabehandling. Ringa in det alternativ i respektive rad i tabellen som stämmer för din hund sedan första behandlingen med cytostatika.

Effekter	Inga biverkningar	1	2	3	4
Aptit		Minskad aptit under ≤ 1 vecka.	Minskad aptit under 1-2 veckor.	Minskad aptit >2 veckor.	-
Anorexi		Ej ätit på <48 h.	Ej ätit på 2-3 dagar.	Ej ätit på 3-5 dagar med påtagligt minskad vikt.	Ej ätit på >5 dagar med påtagligt minskad vikt.
Förstoppning		Tillfälliga förstoppningssymtom. Tillfälliga behov av laxeringsmedel eller behov av att ändra kosten.	Ihållande förstoppningssymtom Regelbundet behov av laxeringsmedel.	Förstoppningssymtom som påverkar hundens dagliga aktivitet. Behov av veterinärhjälp.	-
Diarré		Minskad konsistens på avföringen eller mild diarré som varar under ≤24 h som löser sig själv.	Diarré >24 h.	Diarré som kräver inskrivning på sjukhus och som påverkar daglig aktivitet (äta, dricka, sova, urinering, avföring).	-
Buksmärta		Inga tecken på buksmärta.	Buksmärta-/kramper *eller* slemmig eller blodig avföring *eller* behov av läkemedel *eller* inskrivning på djursjukhus ≤48 h.	Buksmärta-/kramper *och* feber *eller* inskrivning på djursjukhus i >48 h.	-
Illamående		Salivation eller smaskande, kräver ingen åtgärd.	Salivering eller smaskande, kräver åtgärd.	-	-
Kräkningar		Kräkning ≤24 h, kräver ingen åtgärd.	Kräkning >24 h.	Kräkning som kräver inskrivning på sjukhus *eller* Kräkning som påverkar hundens dagliga aktivitet.	-

Ringa in det alternativ i respektive rad i tabellen som stämmer för din hund sedan första behandlingen med cytostatika.

Effekter	Inga biverkningar	**1**	**2**	**3**	**4**
Svårighet att svälja		Lite svårt att svälja men kan äta normal mat.	Påverkat ätbeteende, kräver t. ex. förändrade matvanor eller förändrad foderkonsistens.	Kraftigt påverkat ätbeteende, behöver behandlas på djursjukhus.	-
Fisar		Normalt.	Mer än vanligt.	Mycket mer än vanligt.	-

Andra biverkningar från mage/tarm som ej listats ovan	Fritext: _____ _____ _____ _____ _____ _____

Tack för ditt deltagande!

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file. If you are more than one author, the checked box will be applied to all authors. Read about SLU's publishing agreement here:

- https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

☒ YES, I hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.